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IDENTITY OF THE HYDROLYTIC BASE OBTAINED FROM *DELPHINIUM BROWNII* RYDB. WITH LYCOCTONINE[1]

BY LÉO MARION[2] AND R. H. F. MANSKE[3]

Abstract

The base obtained by hydrolysis of the chief alkaloid present in *Delphinium Brownii* Rydb. is shown to be identical with lycoctonine by comparison with an authentic sample of the latter. The perchlorate, picrate, and methiodide of lycoctonine from both sources have been compared and found to be identical.

Delphinium Brownii Rydb. has been shown to contain an alkaloid that on hydrolysis gives rise to methylsuccinic acid, anthranilic acid, and a crystalline base, the possible identity of which with lycoctonine was suggested (2). Lycoctonine was obtained by Schulze and Bierling (3) as a product of hydrolysis of the amorphous alkaloid lycaconitine isolated from *Aconitum lycoctonum*. More recently, Goodson (1) isolated from *Delphinium elatum* L. an alkaloid that on hydrolysis yielded methylsuccinic acid, anthranilic acid, and a base that he claimed was identical with lycoctonine, although he had no sample of authentic lycoctonine for comparison and failed to prepare any derivative from his base.

The hydrolytic base obtainable from *D. Brownii* has now been further studied and four of its salts have been prepared together with the methiodide. For purposes of comparison lycoctonine has been prepared by the hydrolysis of amorphous lycaconitine isolated from *Aconitum lycoctonum*. Lycoctonine together with its perchlorate, picrate, and methiodide are identical with the hydrolytic base obtained from *D. Brownii* and the corresponding salts and methiodide of this base. No depressions in melting points were observed upon admixture of the two bases or their corresponding salts. This result affords the first definite and unequivocal proof of the occurrence of lycoctonine in species of *Delphinium* and of *Aconitum*.

Since the empirical formula of lycoctonine has never been satisfactorily established, the hydrochloride and hydrobromide have been prepared besides the derivatives already mentioned. The analytical figures obtained from the base, its methiodide, and perchlorate are in best agreement with the formula $C_{20}H_{33}O_6N$. The analysis of the hydrochloride and the hydrobromide indicate

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that these two salts both crystallize with one mole of methanol. The picrate, however, is a salt of a complex nature that seems to involve two moles of the base per mole of picric acid. It has not been possible to arrive at any rational formula for the picrate. It is noteworthy that lycoctonine and all its derivatives are very difficult to analyse and the agreement between the analytical figures obtained and the calculated values in some cases is not too close. The formula $C_{20}H_{33}O_5N$, until substantiated by the characterization of simpler degradation products, is proposed only as that which fits best the numerous analytical data obtained from the base and its derivatives.

Experimental

Lycoctonine (from D. Brownii)

The hydrolytic base (2) was repeatedly recrystallized from aqueous methanol from which it separated as colourless, soft needles that sintered at 120° C. and melted at 139° C.* Found: C, 62.61; H, 8.84; N, 3.54; 3 OCH_3, 23.61%. Loss on heating *in vacuo* at the temperature of boiling toluene: 4.68%. Calc. for $C_{20}H_{33}O_5N \cdot H_2O$: C, 62.34; H, 9.09; N, 3.64; 3 OCH_3, 24.10; water of crystallization, 3.79%.

The perchlorate of the base was prepared in methanol with the aid of 65% perchloric acid and recrystallized twice from methanol–absolute-ether from which it was obtained as small, colourless prisms, m.p. 215° C. Found: C, 52.63; H, 7.59; N, 2.45; Cl, 7.14%. Calc. for $C_{20}H_{33}O_5N \cdot HClO_4$: C, 51.34; H, 7.27; N, 2.99; Cl, 7.59%.

When the base was treated with an acetonic solution of picric acid and the resulting solution diluted with absolute ether, the picrate crystallized in clusters of lemon-yellow needles. After recrystallization from acetone–ether, it melted at 154° C. Found: C, 53.99, 53.95; H, 6.73, 6.56; N, 6.11, 6.35%.

The hydrochloride was prepared from a solution of the base in methanol by the addition of a few drops of hydrochloric acid. When the resulting solution was evaporated to dryness under reduced pressure, it left a gummy residue, which was dissolved in a little dry methanol, and the solution diluted with ether just short of incipient turbidity. This solution, after standing in the cold for several days, deposited the hydrochloride as colourless, prismatic needles which, when recrystallized twice from methanol–ether, melted at 145° C. with effervescence, after shrinking at 104 to 105° C. Found: C, 57.48; H, 9.16; Cl, 7.20%. Calc. for $C_{20}H_{33}O_5N \cdot HCl \cdot CH_3OH$: C, 57.86; H, 8.72; Cl, 8.15%. Schulze and Bierling (3) report the melting point of lycoctonine hydrochloride as 75° C. (uncorr.). They do not mention having recrystallized the salt, which was undoubtedly impure.

The hydrobromide was prepared with hydrobromic acid by the same procedure used for the hydrochloride. It separated from methanol–ether as small, colourless prisms that shrank slightly at 225° C., turned black at

* *All melting points are corrected.*

about 260° C. and did not melt even at 360° C. Again, this is not in agreement with Schulze and Bierling (3) who give m.p. 88 to 89° C. for lycoctonine hydrobromide. Found: Br, 16.36%. Calc. for $C_{20}H_{33}O_5N$. HBr . CH_3OH: Br, 16.67%.

Lycoctonine methiodide

The methiodide was prepared from the base (3 gm.) dissolved in methanol (25 cc.) containing an excess of methyl iodide by heating in a sealed tube at 100° C. for 1.5 hr. The contents of the tube was evaporated to a small volume and hot ethyl acetate (5 cc.) added. On cooling, the methiodide was deposited as glistening yellow needles which, when recrystallized twice from methanol–ethyl-acetate, melted at 187° C. with some sintering at 184° C. Wt., 4 gm. Found: C, 49.53; H, 7.36; N, 2.32%. Calc. for $C_{20}H_{33}O_5N$. CH_3I: C, 49.51; H, 7.06; N, 2.75%.

Crude Lycaconitine

Aconitum lycoctonum L, grown in a local garden, was dried and ground (dry wt., 2700 gm.). It was extracted with methanol in Soxhlets, the solvent largely distilled, and the residual syrup diluted with water and made acid to Congo with aqueous oxalic acid. After heating 24 hr. on the steam-bath the mixture was cooled and kept in the refrigerator for several days. It was filtered rapidly and the treatment repeated with the insoluble cake. The combined aqueous filtrate was thoroughly extracted with ether, basified with ammonia, and exhaustively extracted with chloroform. The chloroform extract was distilled to dryness on the steam-bath and the residue redissolved in an aqueous solution of oxalic acid. The resulting solution was filtered through a layer of charcoal, extracted with several portions of ether, basified with ammonia, and again repeatedly extracted with ether. This last extract, when distilled on the steam-bath to remove the solvent, left a residue consisting of a dark gum that weighed 9 gm. Extraction of the aqueous solution with chloroform yielded a further 2.5 gm. of base.

Isolation of Lycoctonine

The alkaloid obtained from the ether extract (9 gm.) was dissolved in 95% ethanol (60 cc.) and *N*/1 sodium hydroxide (30 cc.) added to the warm solution, which was kept on the steam-bath until most of the ethanol had evaporated. A little water was then added and the solution allowed to stand at room temperature overnight. Next morning the lycoctonine had crystallized out in clusters of fine colourless needles accompanied, however, by some gummy material. Warm acetone dissolved the lycoctonine but not the gum. Dilution of the acetone solution with water caused the base to crystallize in soft, silky needles that sintered at 120° C. and melted at 139° C. The total weight of purified lycoctonine was 2 gm. In admixture with the hydrolytic base obtained from *D. Brownii* it sintered at 120° C. and melted at 139° C. Found: C, 62.71; H, 9.30; N, 3.45%.

Some of the lycoctonine was converted to the perchlorate exactly as already described. After two recrystallizations from methanol–ether the salt melted

at 215° C. either alone or in admixture with the perchlorate of the *D. Brownii* hydrolytic base. Found: C, 52.28; H, 7.51%.

The picrate of lycoctonine (m.p. 152° C.; found: C, 53.98, 54.10; H, 6.72, 6.64%) and the methiodide (m.p. 188° C.; found: C, 50.27; H, 7.40%) were prepared and found to have the same properties and melting points as the corresponding derivatives obtained from the hydrolytic base isolated from *D. Brownii*. In each case admixture with the corresponding compound failed to depress the melting point.

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THE SOURCE OF ACIDITY OF FRESH MILK FROM JERSEY COWS[1]

By Allen D. Robinson[2] and Herbert S. Samson[3]

Abstract

Thirty samples of Jersey milk were analysed for each of titratable acidity, free carbon dioxide, carbonates, citric acid and citrates, casein, lactalbumin and lactoglobulin, and inorganic phosphates. Partial correlation coefficients were calculated for titratable acidity with each of the other variables. The only significant one was that between titratable acidity and inorganic phosphates. Therefore it is concluded that inorganic phosphates are the primary cause of the acidity of fresh milk.

The titratable acidity of milk, expressed as percentage lactic acid, is a standard test in many laboratories. Very high values are attributed usually to souring. It is recognized, however, that fresh milk has an appreciable titratable acidity of its own and that wide variations in it do occur. Obviously, titratable acidity is due in part at least—in the case of fresh milk it must be due wholly—to some normal constituent or constituents whose varying percentages induce fluctuations in the acidity value.

Larger variations are encountered between the milks of individual cows than between those of herds or between the mixed milks of regions. Values for milks from individual cows have been found by Rice and Markley (24) to be as low as 0.086% and by Caulfield and Riddell (6) to be as high as 0.295%. Reported figures for herd averages agree with those of Golding, Mackintosh, and Mattick (14) who found the acidity of samples of mixed milk taken weekly over a three-year period from 10 Shorthorn cows to range from 0.14 to 0.18% with a yearly average of 0.16%. These differences may be due to any of several factors. Houston (16) lists them as health of the cow, feed, age, breed, stage of lactation, interval of milking, and even the quarter of the udder from which the milk is drawn. Indeed, composition and acidity may vary during the course of a single milking. The effect of stage of lactation on titratable acidity is especially marked. Colostrum has the highest value. This drops rapidly during the first eight weeks of lactation to a fairly constant level. A further noticeable drop is observed towards the end of the lactating period. Houston (16), and Caulfield and Riddell (6), and others have stressed the effect of this factor.

A suggestion that acidity is carried over into the milk from an acid in the animal's food appears to be definitely disproved. McNab (20) found that the acidity of the milk from silage-fed cows was no greater than that of milk from cows receiving no silage. Sommer and Hart (27) included sulphuric

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[3] *Graduate Student.*

acid in the ration to the extent of 120 cc. of concentrated acid daily for six days without affecting the acidity of the milk. Duncombe (9) fed cattle lactic, butyric, and phosphoric acids without altering the acidity of their milk. Anderson, White, and Johnson (1) found that corn-gluten feeding does not affect the titratable acidity. McInerney (19) found no relation between the kind of feed and the percentage of acid in the miik.

It has been emphasized by several investigators that the titratable acidity of milk is not a measure of the actual amount of acidity or hydrogen ion concentration of the milk. It is rather a measure of the buffer capacity of some milk constituents and their ability to resist the effect of added alkali on the milk between its normal pH and that of the turning point of the indicator—usually phenolphthalein. Vandevelde (30) calls the degree of acidity obtained by such a volumetric titration an arbitrary figure, while Grimes and Lyons (15) point out that low acidity milks may grade from good to very bad as judged by other tests. Davis (7) states that since the magnitude of the titration is affected by the total solid content of the milk—that is, its richness—a high titratable acidity may indicate a rich milk. Indeed a milk with a low total solids content may be slightly soured and yet give a normal titratable acidity.

High total solids and a high ash content have been associated with high titratable acidity in fresh milk by McInerney (19). Roeder and Radoi (25) found such a relation only when the composition of the total solids remains the same. Of the various milk constituents that might be responsible for the effect, particular attention has been directed to carbon dioxide, citric acid, casein, and inorganic acid phosphates. It has been claimed for each of these that it contributes in a greater or lesser degree to the titratable acidity of milk. Rice and Markley (24), using the results of analyses of 13 samples together with data reported in the literature, formed the constituents into hypothetical combinations. They concluded that casein contributes about one-half of the total titratable acidity; citrates, carbon dioxide, and lactalbumin, a small portion; and phosphates, the balance. They also advance the hypothesis that a condition of equilibrium exists between the citric acid, phosphoric acid, casein, and the bases, therefore the acidity due to any one of these depends upon its relation to the others. Sommer (26) concludes that the acidity of fresh milk is due principally to the phosphates and proteins in milk and to a lesser extent to the carbon dioxide and citrates. Wiley (32) prepared the various buffers in concentrations approximately equal to those in which they appear in milk and studied their buffer effects over different pH ranges. He found that between pH 6.6 and 8.0 phosphates and citrates increase titration with sodium hydroxide, while casein increases it slightly and calcium decreases it. He emphasized "the interdependence of the buffers, phosphate, citrate, casein and calcium."

Carbon dioxide may contribute somewhat to the acidity of milk. Van Slyke and Baker (28) found that the amount of carbon dioxide in milk and its titratable acidity decreased together in a general way. McDowall and

McDowell (18) found a general but not regular correspondence between acidity and the carbon dioxide content of cream. Peter (23) found that the acidity of milk decreased with the time and temperature of heating. He attributed this in part to the loss of carbon dioxide from it and in part to changes induced in casein by heating.

Giza (13) attributes the buffer action of milk chiefly to casein. So do Bordas (3) and Bordas and Touplain (4) who tested separately milk serum, the coagulum containing the insoluble salts, casein plus insoluble salts, and pure casein. Whittier (31) emphasizes, too, the importance of casein because of the similarity between the buffer curve for casein and those for milk.

Buchanan and Peterson (5) found that the titration curves of milk and disodium phosphate solutions are quite similar and conclude that phosphates are at least among the most important buffers of milk. Van Slyke and Bosworth (29) attribute the acidity to acid phosphates. This is supported by another kind of evidence. Olson (22, pp. 30-31) found that the phosphorus content of milk is high at the beginning of lactation, drops to a normal level in about six weeks and decreases gradually until the end of the lactation period. Reference has already been made to titratable acidity following the same course. Jacobsen and Wallis (17, pp. 5-28) found a decrease in the phosphorus content of milk during the first six months of lactation, after which time it increased steadily. However, they found that the values for Jersey milk were higher than those for milk from other breeds. This is known to be true in the case of titratable acidity.

All of this suggests that casein and inorganic phosphates may be responsible for most of the titratable acidity of milk. Carbon dioxide, citric acid, and lactalbumin may contribute in small measure or not at all to this property. Normal variations in casein and phosphate content of milk would account for variations in titratable acidity.

Probably we should distinguish between those substances that are primarily responsible for the titratable acidity and those that happen to be present in the milk as free acids. That there are several different free acids present may be demonstrated by routine analysis. And since they are free acids they will react with added alkali. But some of them may owe their presence to the fact that they are end-products of buffer activity and not to their being the original acids of the milk. Thus an acid phosphate might be buffered by a carbonate to form a secondary phosphate and carbonic acid. The carbonic acid would be the free acid of such a system and would react with any alkali added to it. But the acid phosphate would be the substance primarily responsible for the acidity of the system.

Information on the primary cause of acidity in fresh milk might be obtained by studying the composition of a series of milks and determining the partial correlation coefficient for each of its constituents with titratable acidity. The analyses should take note of all the constituents that might contribute to acidity, namely, carbon dioxide, carbonates, citric acid, citrates, casein, lactalbumin, lactoglobulin, and phosphates. If any of these constituents

should show a significant partial correlation coefficient with titratable acidity, then it might be expected to contribute to the acidity. If the coefficient for any constituent is too small to be significant then that constituent could hardly be the cause of the acidity. We are justified in considering salts like citrates and carbonates since, as we have pointed out above, they may have been formed in the buffering of the original acid. As a matter of fact, while we speak of the proteins as though they are present in milk in the free form they are present for the most part as protein salts.

Experimental

The milks selected for study were from individual purebred Jersey cows. These were chosen because they were known to have higher titratable acidities than normal. An acidity value as high as 0.20% had been noted for the herd. Individual cows had been found to give milks with acidities varying from 0.14 to 0.27%. No attempt was made to select milk from cows in any particular stage of lactation. Rather they were taken at random from the herd. Each sample consisted of a quart of milk taken from the mixed complete morning milking of the individual cow, capped to prevent loss of carbon dioxide, and kept at icebox temperature until analysed. The analyses for carbon dioxide and titratable acidity were made on the fresh milk within three hours of milking. Samples were then preserved by the addition of 1 cc. of 36% formaldehyde and held in the icebox for further analyses.

Titratable acidity was determined by titrating a 50 cc. sample of milk with $N/10$ sodium hydroxide solution, using 10 drops of 1% phenolphthalein in alcoholic solution as the indicator. These quantities are several times those commonly used and should reveal slight differences between the milks.

Free carbon dioxide was determined by an adaptation of the method indicated by Faulk (10, pp. 226-228) for the determination of carbon dioxide in inorganic substances. The method, in brief, consists of releasing the carbon dioxide from milk by boiling, passing the gases through drying agents, and collecting the carbon dioxide in a weighed caustic potash absorption bulb. Carbonates were determined in a somewhat similar way. To the sample from which carbon dioxide had been removed by boiling, lactic acid was added. The mixture was boiled again for 30 min. The carbon dioxide released by the action of the acid and the heat was collected in a weighed caustic potash absorption bulb. Carbon dioxide is reported in terms of volumes per cent. Carbonates are reported as carbon dioxide in the same units.

Citric acid and citrates were determined by Beau's (2) modification of the method proposed by Deniges (8). Casein was ascertained by the method of Moir (21). Lactalbumin and lactoglobulin were determined together using Moir's procedure. Inorganic phosphorus was determined by analysing the milk serum from which casein, lactalbumin, and lactoglobulin had been removed, using the method of Fiske and Subarrow (12) for the determination of phosphates in blood and urine. These analyses are reported in the terms commonly employed for each.

The results of all the analyses are recorded in Table I. These are for 30 different milk samples. Employing these data the average and standard

TABLE I

ANALYSES OF MILK

Sample No.	Titratable acidity as lactic acid, %	Carbon dioxide, volume %	Carbonates as carbon dioxide, volume %	Citric acid and citrates as citric acid, cgm./litre	Casein, %	Lactalbumin and lactoglobulin, %	Inorganic phosphates as phosphorus, mgm./100 cc.
1	0.180	5.51	0.53	28.0	2.44	0.53	67.9
2	0.190	6.22	1.12	26.5	1.81	0.53	71.4
3	0.154	6.09	0.63	69.5	2.48	0.44	61.9
4	0.183	5.89	0.48	35.5	3.14	0.47	64.3
5	0.165	7.19	0.58	44.5	2.38	0.65	66.2
6	0.169	5.17	0.86	45.7	2.52	0.54	60.0
7	0.140	8.13	0.96	53.0	2.60	0.57	61.7
8	0.158	7.62	0.51	32.5	2.51	0.60	64.3
9	0.176	7.09	0.86	12.0	2.79	0.70	62.7
10	0.151	5.45	0.68	14.7	2.82	0.48	63.5
11	0.176	7.67	0.96	59.0	3.24	0.46	65.9
12	0.176	5.66	0.56	51.5	3.34	0.54	56.5
13	0.169	3.72	0.56	42.7	3.21	0.56	63.7
14	0.183	4.82	0.71	43.0	2.50	0.75	64.8
15	0.187	5.40	0.61	50.0	3.38	0.66	67.1
16	0.187	4.94	0.73	32.5	3.36	0.66	72.6
17	0.183	5.12	0.86	40.0	3.02	0.57	65.7
18	0.205	4.18	0.68	26.5	3.56	0.69	71.6
19	0.174	5.51	0.53	62.0	3.06	0.52	62.0
20	0.193	8.27	0.74	19.0	3.64	0.65	72.3
21	0.160	7.73	0.86	60.5	2.59	0.50	55.9
22	0.162	6.12	0.74	59.0	3.07	0.70	54.2
23	0.187	6.89	0.76	32.5	2.93	0.50	69.9
24	0.140	8.45	0.20	56.0	2.57	0.53	56.3
25	0.165	9.48	0.69	48.5	2.92	0.67	59.0
26	0.190	7.61	0.43	26.5	2.26	0.50	67.3
27	0.153	6.43	0.76	47.2	2.57	0.60	57.2
28	0.165	7.55	0.66	50.0	3.00	0.57	60.9
29	0.173	7.95	0.84	54.5	2.76	0.54	61.7
30	0.155	7.10	0.94	48.7	2.68	0.60	55.1

deviation for each constituent were calculated. Correlation coefficients were obtained using as variables

1. Titratable acidity.
2. Carbon dioxide.
3. Carbonates.
4. Citric acid and citrates.
5. Casein.
6. Lactalbumin and lactoglobulin.
7. Inorganic phosphates.

Partial correlation coefficients for titratable acidity with each of the other variables were calculated. The formulae of Fisher (11) were used throughout. The results of these statistical studies are recorded in Table II.

TABLE II

STATISTICAL STUDY OF MILK COMPOSITION

Constituent	Average	Standard deviation	Correlation coefficient with titratable acidity	Partial correlation coefficient with titratable acidity
Titratable acidity, as % lactic acid	0.1716	0.0157	—	—
Carbon dioxide, volume %	6.499	1.368	−.3799	−.2397
Carbonates, as carbon dioxide, volume %	0.701	0.186	.0856	.0999
Citric acid and citrates, as citric acid, cgm./litre	42.38	14.50	−.4675	−.0467
Casein, %	2.838	0.411	.3552	.2984
Lactalbumin and lactoglobulin, %	0.576	0.080	.2159	.1373
Inorganic phosphates, as phosphorus, mgm./100 cc.	63.45	5.17	.7376	.6213*

** The value of P for this correlation coefficient is less than .01. That is, the odds are greater than 99 to 1 that the correlation is not fortuitous but represents a real relation between the variables.*

Discussion

As indicated in Table II the partial correlation coefficient of titratable acidity and inorganic phosphate is significant. The probability against its being a chance happening is more than 99 to 1. However, the partial correlation coefficients of acidity–carbon dioxide, acidity–carbonate, acidity–citric acid and citrates, acidity–casein, and acidity–lactalbumin and lactoglobulin are too low to be considered significant. The probability that each of these values is the result of random sampling is greater than .1.

The value of .6213 obtained as the partial correlation coefficient of titratable acidity and inorganic phosphate is quite high. Ordinarily 30 is not a large number of samples to analyse for purposes of statistical analysis. A high correlation is necessary to indicate a relation between the two variables. The probability of chance happening in this case is less than .01. The positive values indicate a direct relation. When individual values are plotted on graph paper the relation is shown to be linear. These findings show that the inorganic phosphates of the Jersey milks examined are responsible for their titratable acidities. The titratable acidity of fresh milk of other breeds may be caused by the same constituent.

It is not likely that the titratable acidity of fresh milk is due entirely to its phosphate content. Indeed the analyses reported here show varying percentages of other acid substances in milk. A part of these must exist as free acids which are neutralized in the titration of the milk. It has already been suggested that the presence of such constituents may be the result, not the cause, of acidity: that is, they are end-products of reactions between buffers and stronger acids. Furthermore, the results of other researches to which reference has been made confirm that an equilibrium of several buffer con-

stituents does exist. However, the results of this experiment indicate that inorganic phosphates are the primary cause of the titratable acidity of fresh milk.

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THE EFFECT OF SOLVENT AND TEMPERATURE ON THE VISCOSITY OF THE POLYSACCHARIDE OF IRISH MOSS AND THE EFFECT OF SOLVENT ON ITS INITIAL GELATION[1]

By F. A. H. Rice[2]

Abstract

The viscosity of aqueous extracts of Irish moss (*Chondrus crispus*) has been found to depend mainly on the concentration of the neutral salts present in the solution. Samples of dialyzed polysaccharide, however, show considerable variation. The relation between the *specific viscosity* and the *concentration* in dilute aqueous solutions and formamide is such that $\eta_{sp} = ac_v + bc_v^2$, where η_{sp} is the *specific viscosity*, c_v the *concentration*, and both a and b are constants. In aqueous solution these constants are decreased by the addition of neutral salts. A rise in temperature causes a decrease in viscosity. This decrease is less rapid for solutions in formamide and aqueous solutions containing neutral salts. The effectiveness of individual salt solutions in promoting a decrease in viscosity coincides with their effect in producing precipitation of the polysaccharide. The order of decreasing effectiveness was: potassium chloride, calcium chloride, sodium chloride. When the concentration of gelose is such that gelation takes place on standing, measurements of viscosity become irregular and are not reproducible. The concentration of gelose at which the regular viscosity measurements are obtained decreases with increasing concentration of neutral salts. The rigidity of the gels so formed also decreases. The firmness of a gel seemingly depends on the concentration and type of neutral salt, as well as on the *intrinsic viscosity* of the polysaccharide. The intrinsic viscosity is defined as: the limit of the specific viscosity as the concentration approaches zero.

Introduction

It is well known that the viscosity of a solution of large molecules is dependent on the average particle size of the solute. This has been observed in such polymers as cellulose acetate, polyisobutylene, polyvinyl chloride, polystyrene, and natural rubber (1, 4, 5, 12, 14). The viscosity is also influenced by the solvent and the temperature at which the measurements are made. For example, the viscosity of a solution of rubber in benzene is decreased 40% by the addition of 15% methanol (6).

Various attempts have been made to show a relation between the viscosity and the average particle weight without any positive success. Recently, however, the theory of the solution of moderately dilute solutions of high polymers originated by Guth and Gold (7) and Simha (18) has been expanded by Huggins (9, 10, 11) with the result that the *specific viscosity*, η_{sp}, divided by the volume concentration c_v is known to be given by the equation: $\frac{\eta_{sp}}{c_v} = [\eta] + k'[\eta]c_v^2$, where $[\eta]$ is the limiting value of $\frac{\eta_{sp}}{c_v}$ for zero concentration and k' is a constant characteristic of the system. The *specific viscosity* is defined as: $\frac{\eta_c}{\eta_o} - 1$, where η_c is the viscosity of the solvent–solute system and

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η_o the viscosity of the solvent. More simply we may write the relation as $\eta_{sp} = ac_v + bc_v^2$. The same result has been obtained empirically by Schulz and Blaschke (17). In the formula: $\eta_{sp} = ac_v + bc_v^2$ the constants are found to be affected by both solvent and temperature (13). Theoretically $[\eta]$ should be independent of the solvent and related to the molecular weight, but this has been seldom realized in practice.

The polysaccharide of Irish moss, often referred to as gelose or carrageen, is highly viscous in aqueous dispersion and forms a stiff gel in a concentration of about $2\frac{1}{2}\%$ (8). Companies using Irish moss have found that the viscosity and the concentration at which a gel forms, as well as the resultant firmness of the gels at equal concentrations, differ widely with individual samples. This was confirmed on a large number of samples prepared by the author.

All samples of undialyzed gelose contain various amounts of the salts present in sea water, principally sodium chloride. In addition gelose can be salted out of solution (8), and it has been shown that alkali and alkaline earth salts will increase the firmness of the gels (15, 16). Accordingly it was thought that a study of the effect of several common, neutral salts on the viscosity and gel-forming properties of gelose might help to explain the observed variations in these physical properties, which are being used extensively in various commercial preparations in the food and pharmaceutical industries.

Experimental

The gelose used was prepared from samples of Irish moss collected and bleached in Prince Edward Island. The sample, after being washed for 12 hr. in cold running water, was extracted in distilled water for six hours on the boiling water-bath. The extract was filtered through Hyflo Super-Cel (Johns Manville) on a Büchner funnel, concentrated at 95 to 100° C., precipitated in four volumes of ethanol, and, after redissolving in distilled water, dialyzed for 48 hr. against frequent changes of distilled water. The solution was then concentrated *in vacuo* at 35 to 40° C., and again precipitated in 85 to 90% ethanol. The extract was dried by successive changes of absolute ethanol and anhydrous ether and kept in a vacuum desiccator over fused calcium chloride. The product, which was of a fibrous nature, was ground in a mortar to give a fine, snow-white powder.

Viscosities were measured by means of a Stormer viscosimeter. The weight used was such that the time measured by a stop watch reading to 0.1 sec. ranged between 30 to 40 sec. The solutions were maintained at a temperature of 30° ± 0.5° C. in a bath.

Neither the *intrinsic* nor the *specific viscosity* of different samples of gelose at any particular concentration was uniform when treatment in moistening agent, time of bleaching, and drying previous to extraction varied, although the conditions of extraction were the same. Fig. 1 shows the plot of *specific viscosity* versus the volume concentration for three representative samples. Samples 1, 2, and 3 were exposed to sunlight for increasing lengths of time.

The samples studied showed that at concentrations below about 7 gm. per litre, η_{sp} bore a linear relation to c_v. Above 7 gm. per litre the line became progressively curved in the direction of the y-axis.

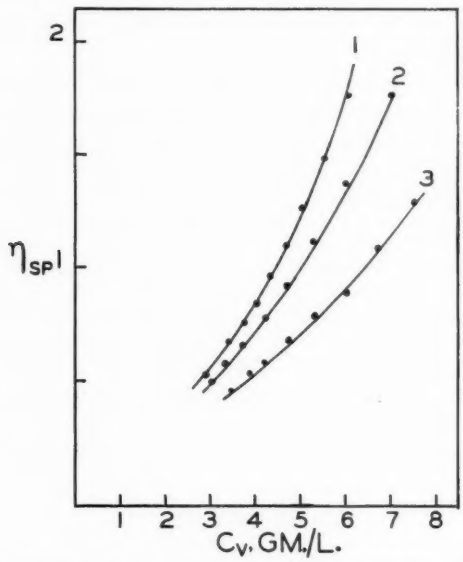

FIG. 1. *The relation of specific viscosity to concentration for three different extracts of gelose.*

To determine the effect of solvent on the viscosity of a gelose solution, samples were dissolved in the solvents indicated in Fig. 2 and their viscosities determined at a number of concentrations. It will be seen that in each case η_{sp}/c_v is linearly related to c_v. The constants a and b, of which a is the value obtained by extrapolation to zero concentration and b is the tangent of the angle made with the x-axis, are decreased in solvents other than distilled water. The solvent affects b to a greater extent than a. At equal concentrations potassium chloride is more effective than sodium chloride. The effect of calcium chloride is not shown but it was midway between the effects of potassium and sodium salts at equal concentrations. Formamide is intermediate in effect between 0.01% solutions of potassium and sodium chlorides.

The solvent also modified the decrease in viscosity due to increased temperature. Measurements made between 25° and 50° C. at concentrations of 1, 3, and 5 gm. per litre of gelose show a progressive loss in viscosity as the temperature increases that is more marked in distilled water than in either formamide or 0.03% sodium chloride solution. Table I shows the results obtained in a 1 gm. per litre solution of gelose. The viscosity at any temperature is decreased in the order: distilled water, formamide, 0.03% sodium chloride solution. The rate of decrease is reduced in the same order.

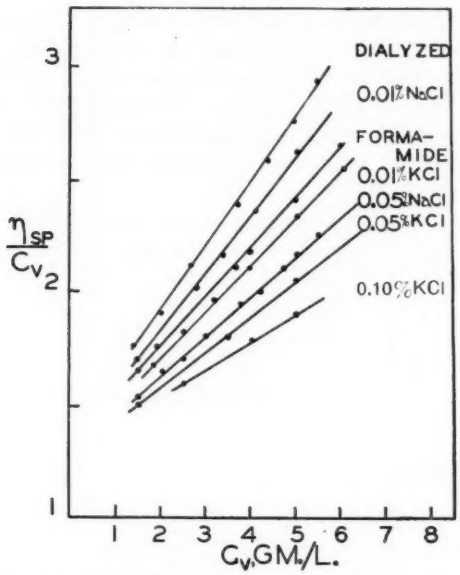

FIG. 2. *The effect of solvent on the viscosity of gelose solutions.*

TABLE I

EFFECT OF TEMPERATURE ON THE VISCOSITY OF GELOSE IN SEVERAL SOLVENTS

Temperature, ° C.	η_{sp} in:		
	Distilled water	Formamide	0.03% NaCl
25	5.78	5.50	4.25
30	4.89	4.51	3.55
35	3.99	3.60	2.95
40	3.05	2.82	2.21
45	2.10	1.99	1.53
50	1.38	1.12	0.96

When the concentration of a gelose solution was increased to a point where gelation would occur on standing (usually about 20 gm. per litre) measurement of viscosity became irregular and could not be reproduced. If neutral salts were added to a gelose solution too dilute to form a gel ordinarily, the viscosity was lowered and a critical concentration of salt could be reached at which the measurement of viscosity became irregular and if left for a time the solution would gel. These gels were very weak and formed only above a concentration of about 5 gm. per litre of gelose. Below this concentration of gelose the polysaccharide was precipitated from solution as a gelatinous precipitate.

Fig. 3 shows the effect of adding 0.06 and 0.25% of potassium chloride to an extract that would ordinarily gel at 19 gm. per litre. It will be seen that gelation was induced at the lower concentration of 7.5 and 6.5 gm. per litre, respectively, by the addition of the salt. The same effect was observed with sodium and calcium chlorides to a lesser degree. The gels formed at these low concentrations were very weak and apparently their firmness depended on the concentration of gelose.

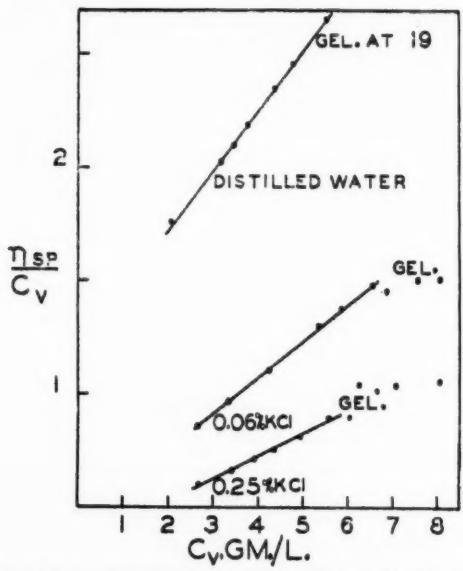

FIG. 3. *The effect of increasing concentrations of potassium chloride on the initial gelation of a gelose solution.*

The *intrinsic viscosity* of the gelose solution also affected the point at which initial gelation occurred. Three samples (Fig. 4) showing different viscosities were treated at a concentration of 6 gm. per litre with sufficient potassium chloride to form a 0.1% solution. It will be seen that the gels first formed at approximately 6, 7, and 8 gm. per litre, the more viscous solutions showing an earlier formation of the gel induced by the same concentration of potassium chloride. If the concentration of the gelose in solution was increased to about 20 gm. per litre in each case, the firmness of the gels was easily seen without measurement to be parallel to the original viscosities of the solutions.

Table II shows the approximate concentration necessary to precipitate the gelose from solutions containing 5 and 2.5 gm. per litre, using potassium, sodium, and calcium chlorides.

Fifty-milligram quantities of the indicated salts were added to 10 ml. of the gelose solution until precipitation took place. The salt concentration was calculated from the amount added.

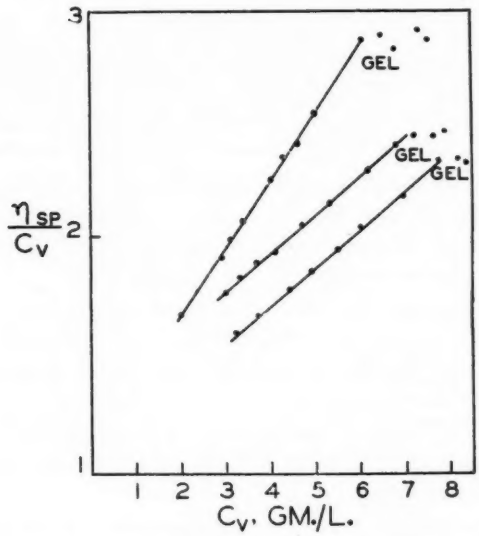

FIG. 4. *The effect of a concentration of 0.1% of potassium chloride on the initial gelation of three gelose solutions of different viscosities.*

TABLE II

CONCENTRATION OF NEUTRAL SALTS REQUIRED TO PRECIPITATE GELOSE FROM SOLUTION

Conc. gelose, gm. per litre	Conc. of salt required to precipitate gelose, %		
	KCl	NaCl	$CaCl_2$
5.0	0.39	1.86	0.42
2.5	0.46	1.84	0.34

It will be seen that the effectivness of the several salts in precipitating the gelose is in the same order as their effect in decreasing the viscosity of the solution.

Discussion

The polysaccharide of Irish moss in dilute solution shows a relation between the viscosity and the concentration that is represented by the equation $\eta_{sp} = ac_v + bc_v^2$. The values of the constants are undoubtedly determined by the average size and shape of the macromolecules and are affected by the solvent in which the viscosity is determined.

Alfrey, Bartovics, and Mark have demonstrated that the viscosity of a polymer is steadily decreased in increasingly poor solvents or solvent–non-solvent mixtures (2). They have also shown that increased temperature

modifies and can in some cases increase the observed viscosity. This is explained in the case of linear polymers as due to the curling of the macromolecule, the degree of which depends on the solubility of the polymer in the medium. In any case, the observed effect is a decrease in the value of the constant b and a lesser decrease for a in the progressively poor solvents.

Huggins (13), using data from the literature on polystyrene and polybutene solutions, has shown that the η_{sp}/c_v values for solutions of a given polymer in different solvents is a function of the proportions of the poor solvent that are required to produce precipitation of the solid phase.

It must be admitted that work of this nature has been done using non-aqueous solvents and not salt solutions, but it is of interest that essentially the same results are obtained with an aqueous solution of gelose if we consider the effect of the added neutral salts to be one of decreasing the solubility of the polysaccharide. That the viscosity of gelose is decreased in formamide, in which it was found to be difficult to dissolve to the extent of 8 gm. per litre, agrees with this hypothesis.

If we consider gelation to occur as a consequence of the precipitation and arrangement of long molecules in a solution, the noted effect of neutral salts in causing earlier gelation is to be expected. The magnitude of the effects of individual salts might be related to their modifying the solubility of the gelose. This would correspond to the observed solubility of the gelose in salt solutions. On the other hand, one would expect that the firmness of the gel would be dependent not only on the precipitation and arrangement of the particulates, which would occur on initial gelation, but on the size and type of the macromolecules or particulates themselves. Bartovics and Mark (3) consider that k' might be a measure of the stiffness of the polymer chain. Alfrey, Bartovics, and Mark also suggest that in a mixture of macromolecules that have different chain lengths and different internal architecture (e.g., branching), precipitation takes place according to the $[\eta]$ values rather than the molecular weights (2).

It must also be remembered, however, that gelose contains ionized calcium (8), which undoubtedly would affect the viscosity in salt solutions inasmuch as added salts, such as potassium chloride, would give rise to an equilibrium between the calcium of the gelose and the added metal. This would mean that a salt solution would contain a mixture of the salts of gelose, the properties of which would depend on the individual properties of the components.

Acknowledgments

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A METHOD OF MEASURING THE STABILIZING POWER OF EXTRACTS OF IRISH MOSS[1]

By F. A. H. Rice[2]

Abstract

A method of measuring the stabilizing power of extracts of Irish moss has been developed. This is based on the quantity of calcium oxalate, expressed as grams per gram of dry extract, held in suspension under specific conditions. The logarithm of this quantity plotted against the concentration of gelose gave a straight line relation. Results were accurate within 1%. Condition of measurement required a temperature below 60° C., pH between 5.5 and 8.5, and a low concentration of neutral salts. Results were compared with those obtained with chocolate powder stabilized in milk. An equation $M = K/t^p c^n$ has been developed and verified, in which the constant K is shown to be numerically an index of stabilizing power, and where M is the number of grams of calcium oxalate held in suspension per gram of extract at a concentration of c grams of extract per 100 ml. after centrifuging for time t. K, p, and n are constants: p and n represent the tangents of lines plotted for the logarithms of M against t and c, respectively.

Introduction

Aqueous extracts of Irish moss (*Chondrus crispus*) have recently been used extensively as a stabilizing agent to hold insoluble material in suspension. The largest use occurs in the stabilization of chocolate milk. Thus approximately 0.03% of dried, purified extract in 500 ml. of milk will prevent the sedimentation of 37 gm. of chocolate. The exact concentration required to hold a given amount of chocolate varies considerably with each sample of extract, and some samples, although forming viscous solutions and firm gels, do not possess this property. The degree to which a sample will prevent sedimentation is sometimes known as the "stabilizing power" of the sample. In the case of chocolate in milk it is usually given as the number of milligrams of dried extract required to maintain in suspension a definite quantity of chocolate in a given quantity of milk.

The active agent and main chemical substance present in these extracts is a polysaccharide sulphate sometimes called "gelose" or "carrageen," (1, 4, 6).

Insoluble inorganic salts such as silver chloride, barium sulphate, and calcium oxalate could not be removed completely from a gelose solution, particularly when they had been freshly precipitated in that solution. It was considered that the amount of these salts held in suspension might serve as a measure of the stabilizing power of the extract.

Before attempting to show a definite relation between the amount of an insoluble salt held in suspension and the stabilizing power as measured in other ways, it was necessary to investigate the limiting factors that might affect the rate of sedimentation of the suspended material in a gelose solution.

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Contribution from the Fisheries Research Board of Canada and the Department of Biochemistry, Dalhousie University, Halifax, N.S.

[2] *Demonstrator in Biochemistry, and Temporary Scientific Assistant, Fisheries Research Board.*

Precipitations of silver chloride, barium sulphate, barium oxalate, and calcium oxalate were tried initially. Attempts to filter and weigh the excess barium sulphate and silver chloride proved very difficult. The formation of an oxalate and the estimation of the sediment by titration against potassium permanganate proved relatively simple.

Method

Quantities of a 0.50% gelose solution were pipetted into 15 ml. graduated centrifuge tubes to give the required concentration or series of concentrations between 0.01 and 0.10 gm. per 100 ml. The contents of each tube was then diluted to 8 ml. with distilled water. To ensure complete solution it was warmed at 60° C. and gently agitated. On cooling, 1 ml. of 0.10 *M* calcium chloride solution was added and, after mixing, 1 ml. of 0.10 *M* sodium oxalate solution was run in drop by drop, the tubes being tapped gently to ensure an even distribution of the precipitate. They were allowed to stand 5 to 10 min. and then centrifuged at 2800 r.p.m. for a known length of time. After the supernatent liquid was poured off, the precipitate was washed twice with 0.2% ammonia solution, centrifuging for about five minutes after each washing. The tubes were then drained on filter paper and 3 ml. of 5% sulphuric acid was added. The calcium oxalate was then titrated against *N*/50 potassium permanganate (2). The amount of calcium oxalate held in suspension was obtained by subtracting this figure from a blank containing no gelose. This figure agreed with that calculated from the amounts of calcium chloride and sodium oxalate used.

Experimental

To ascertain the degree of precision of the method four tubes containing 0.5% gelose and four tubes containing 0.02% gelose, to all of which had been added equivalent amounts of calcium chloride and sodium oxalate, were centrifuged for 5 and 20 min. each. It will be seen from Table I that the results are reproducible within about 1%.

TABLE I

REPRODUCIBILITY OF RESULTS

Tube No.	Mgm. per ml. of oxalate suspended			
	After centrifuging 5 min.		After centrifuging 20 min.	
	0.05% gelose	0.02% gelose	0.05% gelose	0.02% gelose
1	6.72	4.67	2.86	2.62
2	6.66	4.64	2.87	2.62
3	6.72	4.61	2.88	2.63
4	6.66	4.67	2.87	2.61

Violent stirring or shaking immediately after the formation of the precipitate of calcium oxalate caused a decrease of more than 10% in the amount suspended. This was also true when the tubes were centrifuged immediately. After standing 15 min. results were relatively constant as shown in Table II.

TABLE II

EFFECT OF STANDING AFTER THE ADDITION OF SODIUM OXALATE

Time, min.	Mgm. per ml. of oxalate suspended	
	0.05% gelose	0.02% gelose
0	4.35	3.64
5	3.76	3.22
10	3.75	3.21
15	3.76	3.22
30	3.75	3.22
600	3.76	3.21

The addition of an excess of calcium chloride and sodium oxalate caused an increased sedimentation which, at a concentration of 0.05% gelose, became measurable when more than 2 ml. of each reagent were used. This increased sedimentation was associated with a partial precipitation of the gelose from solution. When less than 0.25 ml. of each reagent was used there was no measurable sedimentation after centrifuging 10 min.

Changes in temperature up to 60° C. had no measurable effect (Table III). After the addition of the calcium chloride, the tubes were heated for five minutes at the indicated temperature, and the sodium oxalate was added. After another five minutes they were centrifuged for 15 min. and the determination of calcium oxalate was made as before. Above 60° C. irregular results were obtained.

TABLE III

EFFECT OF TEMPERATURE

Conc. of gelose, %	Mgm. per ml. of oxalate suspended		
	22° C.	56° C.	90° C.
0.10	6.60	6.71	6.56
0.06	5.10	5.08	5.13
0.04	4.04	4.06	3.98
0.02	3.08	3.10	3.06

Sodium chloride, potassium chloride, and calcium chloride, when added in excess, increased sedimentation. Various amounts of each salt were added before the addition of the sodium oxalate and the tubes centrifuged for 10 min. The increased sedimentation varied with the amount and the nature of the salt used (Table IV).

TABLE IV

EFFECT OF NEUTRAL SALTS

Conc. of gelose, %	Additional conc. of salt, moles	Mgm. per ml. of oxalate suspended with added		
		NaCl	KCl	$CaCl_2$
0.05	0.05	3.74	3.75	3.77
	0.1	3.74	3.74	3.75
	0.2	3.73	2.26	2.68
	0.3	3.72	2.05	1.98
	0.4	3.72	—	1.28
0.01	0.05	1.60	1.61	1.62
	0.1	1.61	1.60	1.60
	0.2	1.60	1.22	1.34
	0.3	1.21	—	—
	0.4	0.83	—	—

A change in pH between 5.5 and 8.5 had little effect. Above and below this, however, the calcium oxalate held in suspension was decreased. Results are shown in Table V. The pH was adjusted with sodium hydroxide and acetic acid and measured by means of the glass electrode. The concentration of gelose was 0.05% and the time of centrifugation was 11 min.

TABLE V

EFFECT OF PH

pH of solution	3.1	3.8	5.0	5.5	6.5	7.0	8.5	10.4	11.6
Mgm. per ml. of oxalate suspended	1.92	2.43	2.50	3.71	3.74	3.74	3.78	2.45	1.92

By way of comparison, equivalent amounts of barium hydroxide and oxalic acid were used to form barium oxalate under identical conditions as for calcium oxalate. The results were parallel to those obtained with calcium oxalate (Table VI). It should be noted that in this case no sodium chloride was formed by the reaction between the reagents.

TABLE VI

COMPARISON OF BARIUM AND CALCIUM AS OXALATES

Conc. of gelose, %	Barium, mgm. per ml.	Calcium, mgm. per ml.	Ratio barium : calcium
0.10	2.75	3.06	0.89
0.08	2.71	3.01	0.90
0.06	2.58	2.87	0.89
0.04	2.50	2.77	0.90

The amount of calcium oxalate held in suspension at various concentrations of gelose and after various times of centrifugation was determined. Concentrations from 0.01 to 0.1% gelose were used and at each concentration a series was centrifuged from 10 to 45 min. The number of grams of calcium oxalate retained in suspension per gram of gelose after any particular time of centrifugation was found to be logarithmically related to the concentration of gelose (Fig. 1). The same logarithmic relation was observed at any given concentration when the time of centrifugation was varied (Fig. 2).

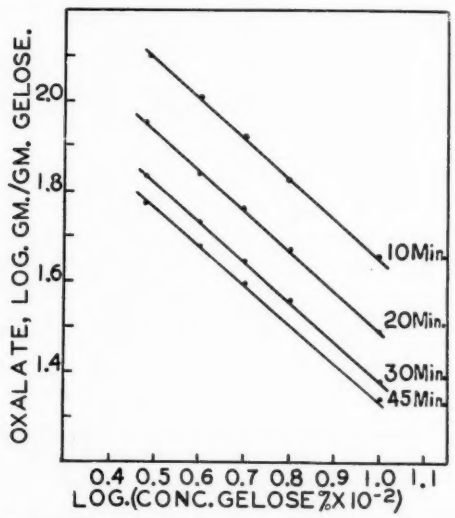

FIG. 1. *Influence of concentration of gelose on oxalate held in suspension.*

A number of samples were tested for this relation and it was found to be true in each case. The tangent of the lines relating the amount of calcium oxalate to the concentration of gelose or time of centrifugation, however, was found to vary with the individual sample. The extrapolated value for zero concentration and zero time of centrifugation also varied with the sample.

Because of this logarithmic relation we may write for any given sample: $M = K/t^p c^n$, where M is the number of grams of calcium oxalate held in suspension per gram of gelose at a concentration c grams of gelose per 100 ml. after centrifuging for time t. K, p, and n are constants: p and n represent the tangents of lines plotted for the logarithms of M against t and c, respectively. K can be obtained by substitution at any concentration where the calcium oxalate in suspension is known.

To check the calculated with the observed values these constants were determined on a sample of gelose. Values were calculated for various concentrations and times of centrifugation and actual measurements made. The results agreed within less than 1% (Table VII).

TABLE VII

AGREEMENT IN OBSERVED AND CALCULATED VALUES

Time of centrifugation, min.	Conc. of gelose, %	Grams oxalate per gram gelose	
		Calculated	Observed
20	0.03	893	892
20	0.05	575	576
10	0.03	1190	1210
15	0.05	671	670
35	0.05	421	428
10	0.10	457	452

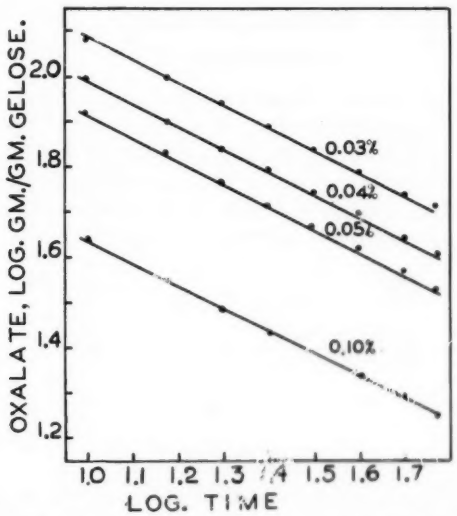

FIG. 2. *Influence of period of centrifugation on suspension of oxalate in gelose.*

A series was directly compared to determine whether a relation existed between the constants obtained with calcium oxalate and the stabilizing power of the gelose towards chocolate in milk. To measure the stabilizing power of different samples of gelose as regards chocolate, 36.6 gm. of chocolate was added to each of a series of bottles containing 0.02 to 0.06% of gelose at intervals of 0.005% in 500 ml. of milk. The milk tested 2% butter fat. The lowest concentration of gelose that retained the chocolate in suspension after 48 hr. was taken as the index of stabilizing power. If the number of grams of chocolate held in suspension per gram of gelose is calculated from the concentration of gelose necessary to stabilize the chocolate, as is done in the case of the calcium oxalate, it is found (Table VIII) that the amount of chocolate is directly related to the constant K within the unavoidable 10% error made in the determination of stabilizing power by means of chocolate.

TABLE VIII

COMPARISON OF CONSTANTS OBTAINED FROM CALCIUM OXALATE APPLIED TO CHOCOLATE IN MILK

Sample No.	K	p	n	Chocolate held in suspension per gram gelose	K/chocolate per gram gelose
2	211	0.525	0.860	162.8	1.38
3	202	0.254	0.748	146.4	1.30
4	7.3	0.217	0.140	<24.4	—
5	4.1	0.790	0.326	<24.4	—
6	247	0.101	0.467	183.0	1.35
7	329	0.428	0.325	244.0	1.35
8	177	0.152	0.620	106.7	1.60
9	215	0.314	0.208	—	—

In the simplest form of the proposed method, constants can be determined by measuring the calcium oxalate retained in suspension at two concentrations of gelose after two different periods of centrifugation at each concentration. Suppose, for example, m_1, m_2 grams of oxalate per gram of gelose are suspended at concentrations c_1, c_2, respectively, after centrifuging t_1 minutes, and m_3, m_4 at the same concentrations after centrifuging a longer time t_2. Then:

$$n = \frac{\log m_2 - \log m_1}{\log c_2 - \log c_1} \text{ or } \frac{\log m_4 - \log m_3}{\log c_2 - \log c_1}$$

$$p = \frac{\log m_1 - \log m_3}{\log t_2 - \log t_1} \text{ or } \frac{\log m_2 - \log m_4}{\log t_2 - \log t_1}$$

At concentration c_1 or c_2, K can be determined from the equation $K = m\, c^n t^p$.

Discussion

From the evidence presented it would seem that the amount of calcium oxalate held in suspension in a gelose solution at various concentrations and after various times of centrifugation can serve to determine several constants which vary with the gelose sample. The exact meaning of these constants is not clear. Ewe (3) has shown that methylene blue will precipitate gelose from solution. He suggested that the dye was adsorbed, forming an insoluble complex. It may be that the observed stabilizing power is an adsorption phenomenon associated with particulates of small diameter.

Adsorption has been found to follow a certain rule in regard to the quantity taken up by a given weight of adsorbent. This is usually stated as $a = Ac^{1/n}$ (the so-called Freundlich adsorption isotherm). If the logarithms of this equation are taken we get $\log a = \log A + 1/n \log c$. The two sides of this equation bear a linear relation to each other. The similarity between this and the effect noted for calcium oxalate in gelose solution is evident.

Mason and Weaver (5) showed that "all suspensions should sediment under the influence of gravity" and "this system should show a logarithmic gradient

of concentration." We might assume that the suspended material would therefore be thrown out of suspension logarithmically under the influence of centrifugal force and hence the material in suspension would be logarithmically depleted.

It is believed that these suggestions might account for the observed relations of stabilization in gelose solutions.

Acknowledgments

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SYNTHESIS OF A NEW α-AMINO ACID, S-METHYL-β,β-DIMETHYLCYSTEINE[1]

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Abstract

A new sulphur-containing α-amino acid, S-methyl-β,β-dimethylcysteine has been prepared from S-benzyl-β,β-dimethylcysteine. These two compounds and the intermediate β,β-dimethylcysteine may be of physiological importance.

S-Benzyl-β,β-dimethylcysteine has been prepared by the addition of benzyl mercaptan to α-benzoylamino-β,β-dimethylacrylic acid, its azlactone, and methyl ester, followed by hydrolysis.

In addition to the above three new sulphur-containing α-amino acids, the following compounds are described, as far as the authors are aware, for the first time: methyl α-benzoylamino-β,β-dimethylacrylate, S-benzyl-N-benzoyl-β,β-dimethylcysteine and its methyl ester, the hydantoin of S-benzyl-β,β-dimethylcysteine, and S-benzyl-β,β-dimethylcysteine ethyl ester hydrochloride, and helianthate.

Introduction

The present study was undertaken in these laboratories to make available for physiological studies the hitherto unknown α-amino acid, S-methyl-β,β-dimethylcysteine. In the synthesis of thiol and thioether α-amino acids, du Vigneaud and co-workers have introduced a convenient approach by first synthesizing the benzylthiol derivative of the desired compound. The benzyl group is then easily removed by reduction in liquid ammonia to give the thiol amino acid (14), or it may be replaced by an alkyl group by reduction and subsequent treatment with the appropriate alkyl halide in liquid ammonia to give the alkylthiol amino acid (4, 5). Accordingly S-benzyl-β,β-dimethylcysteine was prepared by the addition of benzyl mercaptan in the presence of sodium methoxide to the azlactone of α-benzoylamino-β,β-dimethylacrylic acid, followed by hydrolysis. S-Methyl-β,β-dimethylcysteine was then obtained from S-benzyl-β,β-dimethylcysteine by reduction of the latter with sodium in liquid ammonia followed by treatment with methyl iodide.

A study of the addition of mercaptans to α,β-unsaturated ketones and esters has been made by Nicolet (10) and others (cf. Mayo and Walling (8)); the addition of mercaptans to azlactones has also been described by Nicolet (9,11) and Carter, Stevens, and Ney (2). These workers found that addition is catalyzed by alkaline reagents such as piperidine and sodium alkoxides. In the present work the addition of benzyl mercaptan to the azlactone of α-benzoylamino-β,β-dimethylacrylic acid (V) proceeded smoothly in anhydrous medium in the presence of sodium methoxide to give S-benzyl-N-benzoyl-β,

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β-dimethylcysteine methyl ester (IV) in good yield. Addition also took place with both the sodium salt of α-benzoylamino-β,β-dimethylacrylic acid (I) and the corresponding methyl ester (II) to give S-benzyl-N-benzoyl-β,β-dimethylcysteine (III) and the corresponding methyl ester (IV) respectively.

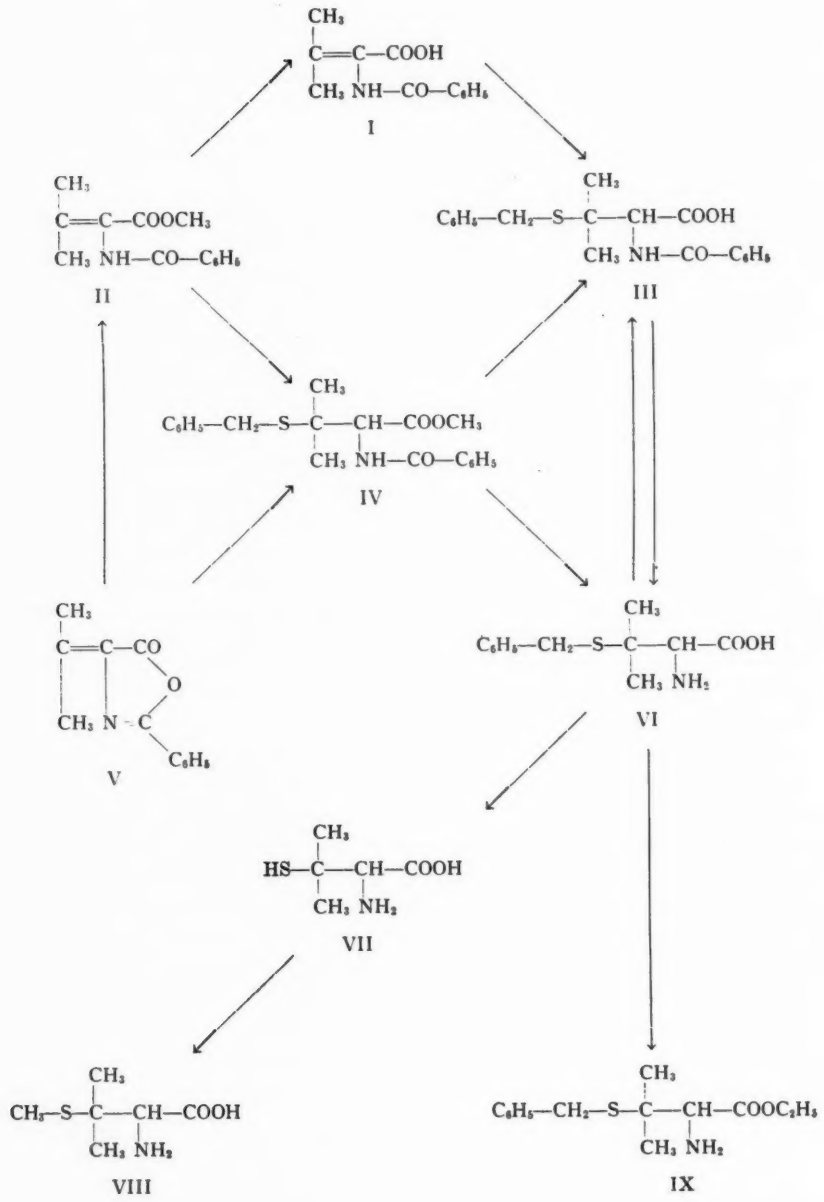
It was found, in the case of addition to the azlactone, that if the conditions cited above were not strictly maintained, or if the purification of the reaction product were delayed (possible prolonged contact with air and moisture), there was isolated from the reaction mixture a considerable quantity of methyl-α-benzoylamino-β,β-dimethylacrylate (II). This compound was also obtained by treating the azlactone (V) with sodium methoxide in anhydrous methanol.

Hydrolysis of S-benzyl-N-benzoyl-β,β-dimethylcysteine methyl ester (IV) to S-benzyl-β,β-dimethylcysteine (VI) could be effected in one or two steps. Because of its insolubility in water, considerable difficulty was encountered in the single step hydrolysis. With most hydrolysing reagents the ester remained unchanged as an oil, even after hours of refluxing, or underwent decomposition; addition of solvents such as alcohol also gave very poor yields. Best results were obtained using a mixture of equal parts of commercial formic acid, concentrated hydrochloric acid, and water (2). Partial hydrolysis with an aqueous mixture of acetic acid and hydrochloric acid gave S-benzyl-N-benzoyl-β,β-dimethylcysteine (III), which, on refluxing with the formic–hydrochloric-acid mixture, gave S-benzyl-β,β-dimethylcysteine (VI). Several derivatives of this compound were prepared, namely, the hydantoin, the ester hydrochloride (IX) and helianthate, and the benzoyl derivative. This last named compound, S-benzyl-N-benzoyl-β,β-dimethylcysteine, was identical with the product obtained by partial hydrolysis of S-benzyl-N-benzoyl-β,β-dimethylcysteine methyl ester (IV).

The replacement of the benzyl group of S-benzyl-β,β-dimethylcysteine (VI) by a methyl group to give the required α-amino acid, S-methyl-β,β-dimethylcysteine (VIII), was accomplished by treating a solution of the above compound in liquid ammonia with sodium according to the method used by du Vigneaud and co-workers (4, 5, 14), and reacting the sodium mercaptide of the corresponding β,β-dimethylcysteine (VII) thus formed with methyl iodide. This hitherto unknown β-thiol-α-amino acid also was isolated as the hydrochloride by precipitation as the water insoluble cuprous mercaptide by means of cuprous oxide (12), followed by decomposition with hydrogen sulphide in dilute hydrochloric acid in the usual manner.

The over-all yield of S-methyl-β,β-dimethylcysteine (VIII), calculated from α-benzoylamino-β,β-dimethylacrylic acid azlactone (V) was 30 to 40% of the theoretical.

It has been found that the β,β-dimethylcysteine inactivates streptomycin, resembling cysteine (3) in this effect. β,β-Dimethylcysteine, when added to the conventional culture medium for the production of penicillin, gave no significant increase in the antibacterial potency of the broth.


I
II
III
IV
V
VI
VII
VIII
IX

Experimental

Methyl α-Benzoylamino-β,β-dimethylacrylate (II)

α-Benzoylamino-β,β-dimethylacrylic acid azlactone (0.40 gm.), prepared according to the directions of Ramage and Simonsen (13), was dissolved in dry benzene (1.6 ml.) and a 5% solution of sodium metal in dry methanol (0.1 ml.) was added. After standing for five minutes crystals appeared; the mixture was then acidified with dilute hydrochloric acid, stirred well, and the crystals collected by centrifuging. Recrystallization from carbon tetrachloride gave white fluffy crystals that sintered at 127° C. and melted at 135 to 136° C. (corrected)*; when mixed with a sample of methyl α-benzoylamino-β,β-dimethylacrylate obtained as a by-product in the preparation of S-benzyl-N-benzoyl-β,β-dimethylcysteine methyl ester, there was no lowering of the melting point.

α-Benzoylamino-β,β-dimethylacrylic Acid (I)·

Methyl α-benzoylamino-β,β-dimethylacrylate (5.0 gm., 0.0214 mole) was refluxed in a mixture of concentrated hydrochloric acid (33.0 ml.), glacial acetic acid (110 ml.), and water (65.0 ml.) for one hour. After evaporating *in vacuo* to one-half volume and cooling, the solution deposited colourless crystals, which were filtered and washed with 50% aqueous acetic acid, followed by water. After one recrystallization from ethyl acetate the colourless crystals had a melting point of 215.5 to 216.6° C. (corrected), which was not lowered when they were mixed with an authentic sample of α-benzoylamino-β,β-dimethylacrylic acid prepared from the corresponding azlactone by hydrolysis with sodium hydroxide (1, 13). The yield was 1.8 gm., corresponding to 38% of theory.

S-Benzyl-N-benzoyl-β,β-dimethylcysteine Methyl Ester (IV)

(a) Prepared from α-Benzoylamino-β,β-dimethylacrylic Acid Azlactone (V)

In a dry apparatus consisting of a three-necked flask equipped with mechanical stirrer, dropping funnel, and gas-inlet tube, sodium metal (0.59 gm., 0.0256 mole) was dissolved in anhydrous methanol (125 ml.) and the apparatus filled with dry nitrogen gas and cooled in ice-water. Freshly distilled benzyl mercaptan (15.5 ml., 0.132 mole) was added, followed by a solution of α-benzoylamino-β,β-dimethylacrylic acid azlactone (25.0 gm., 0.124 mole) in dry benzene (125 ml.), added with stirring over a period of one and one-half hours. (The reaction is slightly exothermic, but may be carried out at temperatures ranging from 0° C. to the boiling point with slight variation in yields.) When the addition was complete, stirring was allowed to continue for one hour, after which the mixture was allowed to stand overnight at room temperature. An exact amount of glacial acetic acid was added, equivalent to the weight of sodium used, and the mixture evaporated to dryness *in vacuo* under 5° C. The residue was dissolved in warm dry benzene (70.0 ml.), filtered, and the insoluble sodium acetate washed with benzene. The benzene filtrate and washings were concentrated *in vacuo* to about 100 ml. and petroleum ether (500 ml.) added. The oil that separated crystallized overnight in the cold.

* *All melting points are uncorrected unless otherwise specified.*

Recrystallized from methanol, the product melted at 66.5 to 67.5° C. (corrected) and weighed 35.0 gm., corresponding to 78.5% of theory. Calc. for $C_{20}H_{23}O_3NS$: C, 67.50; H, 6.49; N, 3.91; S, 8.97%. Found: C, 67.10; H, 6.64; N, 3.86; S, 8.59%.

In several of these runs, where the conditions of the reactions were not rigorously anhydrous, or where the purification of the crude reaction product was delayed, methyl α-benzoylamino-β,β-dimethylacrylate was isolated as by-product. Recrystallized from carbon tetrachloride this product melted at 136.5 to 137.0° C. (corrected) after sintering at 126° C. Calc. for $C_{12}H_{11}O_2N$: N, 5.94%. Found: N, 5.80%.

(b) Prepared from Methyl α-Benzoylamino-β,β-dimethylacrylate (II)

Under the same conditions described in the previous experiment, a solution of methyl α-benzoylamino-β,β-dimethylacrylate (10.0 gm., 0.043 mole) in a mixture (80.0 ml.) of equal parts of methanol and benzene was added to a solution of benzyl mercaptan (5.33 ml., 0.0455 mole) in anhydrous methanol (30.0 ml.) containing sodium metal (0.24 gm., 0.0104 mole).

After standing overnight, the solution was neutralized with glacial acetic acid and the solvents removed *in vacuo*. The residue was dissolved in benzene, filtered, and the reaction product isolated by fractional precipitation with petroleum ether. Three grams of unreacted starting material was recovered and 8.1 gm. of S-benzyl-N-benzoyl-β,β-dimethylcysteine methyl ester was obtained, m.p. 66 to 68° C. The yield was 76% of theory, calculated on the basis of the reacted starting material.

S-Benzyl-N-benzoyl-β,β-dimethylcysteine (III)

(a) Prepared from S-Benzyl-N-benzoyl-β,β-dimethylcysteine Methyl Ester (IV)

S-Benzyl-N-benzoyl-β,β-dimethylcysteine methyl ester (7.14 gm., 0.02 mole) was refluxed for 50 min. in a mixture of glacial acetic acid (60.0 ml.), concentrated hydrochloric acid (18.0 ml.), and water (36.0 ml.), and the resulting solution allowed to crystallize overnight in the cold. The white crystalline product was collected by filtration and washed with aqueous acetic acid; the weight was 4.6 gm., corresponding to 69% of theory. After several recrystallizations from aqueous acetic acid, the S-benzyl-N-benzoyl-β,β-dimethylcysteine melted poorly at 156 to 162° C., after sintering at 151° C. Calc. for $C_{19}H_{21}O_3NS$: C, 66.42; H, 6.18; N, 4.08; S, 9.34%. Found: C, 66.34; H, 6.09; N, 4.28; S, 9.21%.

(b) Prepared from S-Benzyl-β,β-dimethylcysteine (VI)

S-Benzyl-β,β-dimethylcysteine (0.476 gm., 0.002 mole) was dissolved in 2 *N* sodium hydroxide (1.5 ml., 0.033 mole) and water (0.5 ml.), and with stirring, benzoyl chloride (0.43 ml., 0.033 mole) and 2 *N* sodium hydroxide (4.6 ml.) were added simultaneously, and the mixture stirred for 10 min. On acidification with concentrated hydrochloric acid (0.68 ml.) an oil separated which quickly solidified and was filtered. After recrystallization from

50% aqueous acetic acid, 0.50 gm. of S-benzyl-N-benzoyl-β,β-dimethylcysteine was obtained, corresponding to 78% of theory. The melting point was 158 to 163° C., which was not lowered by mixing with a sample of the product obtained in *(a)* above.

S-Benzyl-β,β-dimethylcysteine (VI)

(a) Prepared from S-Benzyl-N-benzoyl-β,β-dimethylcysteine Methyl Ester (IV)

S-Benzyl-N-benzoyl-β,β-dimethylcysteine methyl ester (5.0 gm., 0.014 mole) was refluxed for four hours in a mixture (200 ml.) consisting of equal volumes of 85% formic acid (commercial grade), concentrated hydrochloric acid, and water. The solution was then evaporated to dryness *in vacuo*, and the resulting residue extracted twice with boiling ligroin (65 ml.). The extract was discarded and the residue dissolved in boiling water (70 ml.) and filtered. On adjusting the filtrate to pH 5.8–6.0 a heavy precipitate of S-benzyl-β,β-dimethylcysteine separated; it was filtered and weighed. The yield was 1.8 gm., corresponding to 53% of theory. Recrystallization from water gave white fluffy crystals that melted sharply with decomposition, from 190° C. to 200° C., depending upon the rate of heating. Calc. for $C_{12}H_{17}O_2NS$: C, 60.20; H, 7.16; N, 5.85; S, 13.45%. Found: C, 59.66; H, 6.92; N, 5.53; S, 13.86%.

Later hydrolyses have been carried out using as much as 150 gm. of S-benzyl-N-benzoyl-β,β-dimethylcysteine methyl ester without any decrease in the yield. In these large batches it was found that the crude product resulting from the addition of benzyl mercaptan to α-benzoylamino-β,β-dimethylacrylic acid azlactone could be hydrolysed directly. The substitution of ether for ligroin in the purification of the hydrolysis product gave much better results; in addition to the removal of benzoic acid, all colour and considerable amounts of unreacted S-benzyl-N-benzoyl-β,β-dimethylcysteine ester (which could be recovered) were removed from the final product, the latter requiring but one recrystallization from water to give analytically pure S-benzyl-β,β-dimethylcysteine.

(b) Prepared from S-Benzyl-N-benzoyl-β,β-dimethylcysteine (III)

S-Benzyl-N-benzoyl-β,β-dimethylcysteine (6.40 gm., 0.0186 mole) was refluxed in the hydrolysis mixture described above (90.0 ml.) and the solution treated as in (*a*). After recrystallization from water, 1.6 gm. of S-benzyl-β,β-dimethylcysteine was obtained, corresponding to 36% of theory.

(c) Prepared from α-Benzoylamino-β,β-dimethylacrylic Acid (I)

Metallic sodium (0.470 gm., 0.0204 mole) was dissolved in methanol (30 ml.), and, in an atmosphere of dry nitrogen gas, benzyl mercaptan (2.5 ml., 0.0213 mole) was added. A solution of α-benzoylamino-β,β-dimethylacrylic acid (4.38 gm., 0.020 mole) in methanol (50.0 ml.) was added slowly to the stirred solution and the resulting solution then allowed to stand overnight. Glacial acetic acid (1.15 ml., 0.019 mole) was added and the solvents removed *in vacuo*.

The product, S-benzyl-N-benzoyl-β,β-dimethylcysteine, could not be separated from the starting material by crystallization, so the entire dry reaction residue was refluxed for four hours in a mixture consisting of concentrated hydrochloric acid (95 ml.), 85% formic acid (95 ml.), and water (95 ml.), and the solution evaporated to dryness *in vacuo*. In this hydrolysis any starting material is converted to β,β-dimethylpyruvic acid (13). The dry residue was extracted with boiling ligroin (to remove benzoic acid); the insoluble material was dissolved in dilute hydrochloric acid (80 ml.) and filtered. The filtrate was then adjusted to pH 5.8 and cooled. A small crop of S-benzyl-β,β-dimethylcysteine was filtered and dried; the yield was 20.7 mgm., corresponding to 4% of theory. This compound was identified by melting point, which was not lowered when the compound was mixed with S-benzyl-β,β-dimethylcysteine as prepared in (*a*) above.

S-Benzyl-β,β-dimethylcysteine Hydantoin (5-(β-Benzylthiol-isobutyl)hydantoin)

S-Benzyl-β,β-dimethylcysteine (3.75 gm., 0.015 mole) and potassium cyanate (1.52 gm., 0.02 mole) were refluxed in water (75 ml.) for 45 min. according to the method of Karabinos and Szabo (7). After cooling, the solution was acidified with 10% hydrochloric acid (45 ml). and again refluxed for 30 min. On standing overnight, the solution deposited colourless crystals, which were filtered and dried. Recrystallized twice from ethanol and once from ethyl acetate, the hydantoin weighed 2.6 gm., corresponding to 61% of theory; melting point 204 to 205° C. Calc. for $C_{13}H_{16}O_2N_2S$: N, 10.61; S, 12.14%. Found: N, 10.39; S, 12.32%.

S-Benzyl-β,β-dimethylcysteine Ethyl Ester (IX)

S-Benzyl-β,β-dimethylcysteine (0.50 gm., 0.0021 mole) was suspended in absolute ethanol (25 ml.), cooled in ice-water, and the suspension saturated with dry hydrochloric acid and allowed to stand overnight. After removal of the excess ethanol by evaporation *in vacuo*, the excess hydrochloric acid was removed by standing in a desiccator over solid sodium hydroxide. The dry residue was dissolved in the minimum volume of ethanol and precipitated with dry ether. After several precipitations, 0.63 gm. of the ethyl ester hydrochloride was obtained, corresponding to 95% of theory; the product melted at 157 to 158.5° C., and was very hygroscopic. Calc. for $C_{14}H_{22}O_2NSCl$: N, 4.61%. Found: N, 4.95%.

S-Benzyl-β,β-dimethylcysteine Ethyl Ester Helianthate

S-Benzyl-β,β-dimethylcysteine ethyl ester hydrochloride (0.20 gm., 0.00066 mole) was dissolved in water (2.0 ml.) and treated according to the direction of Gurin and Segal (6) with a hot solution of methyl orange (0.21 gm., 0.000642 mole) in water (5.0 ml.). The reaction was immediate, giving a voluminous bright yellow precipitate of the methyl orange derivative, which after cooling was filtered and washed with a little cold water. Recrystallized from dry methanol, the crystalline helianthate melted at 183 to 183.5° C.,

with darkening. The yield was 0.232 gm. corresponding to 84% of theory. Calc. for $C_{28}H_{36}O_5N_4S_2$: S, 12.21%. Found: S, 12.27%.

S-Methyl-β,β-dimethylcysteine (VIII)

S-Benzyl-β,β-dimethylcysteine (9.22 gm., 0.04 mole) was placed in a dry 500 ml. flask equipped with a side-arm and mercury-sealed stirrer, and the flask immersed in a dry-ice–alcohol bath. Liquid ammonia (300 ml.), which had been dried by collection over sodium metal, was then allowed to redistill into the reaction flask. With stirring, sodium metal was added in small portions until a total of 2.0 gm., corresponding to 2.5 equivalents, was used. The deep blue colouration of the solution, due to a slight excess of sodium, was then discharged with a small amount of ammonium chloride.

Methyl iodide (6.25 gm., 0.044 mole) was added to the solution, and after a short time stirring was stopped, the flask removed from the dry-ice bath, and the ammonia allowed to evaporate through a soda-lime tube and mercury-trap to prevent the entry of moisture; the last traces of ammonia were removed *in vacuo*. The colourless residue was dissolved in cold water (75 ml.), quickly extracted twice with ether (25 ml.) (to remove dibenzyl), and the aqueous solution quickly brought to pH 5.8–6.0 with concentrated hydrochloric acid, and cooled. The white crystals that separated were filtered and washed with cold water; concentration and cooling of the filtrate yielded a second crop of crystals.

Recrystallization of the crude product from water gave 6.1 gm. of pure S-methyl-β,β-dimethylcysteine corresponding to 96% of theory. The melting point varied with the rate of heating; the sample used for analysis melted at 254 to 256° C. Calc. for $C_6H_{13}O_2NS$: N, 8.59; S, 19.65%. Found: N, 8.46; S, 19.60%.

β,β-Dimethylcysteine Hydrochloride (VII)

S-Benzyl-β,β-dimethylcysteine (4.76 gm., 0.02 mole) was added to anhydrous liquid ammonia (300 ml.) contained in a dry flask equipped with a mercury-sealed stirrer and cooled in a dry-ice–alcohol bath. To this mixture metallic sodium (1.098 gm., 0.047 mole) was added in small pieces, until an excess was indicated by a blue colouration lasting 5 to 10 min. The solution was decolorized by the addition of a small amount of ammonium chloride. The liquid ammonia was then allowed to evaporate through a mercury trap, the latter to prevent air oxidation.

The residue was dissolved in water (20 ml.), and the resulting solution cooled, and treated with concentrated hydrochloric acid (2.5 ml.). The solution was then extracted with ether to remove dibenzyl, and evaporated to dryness *in vacuo* under an atmosphere of nitrogen, the temperature being kept below 45° C. The residue was dissolved in water (50 ml.), and an excess of freshly prepared cuprous oxide was added with vigorous stirring. The suspension was adjusted to pH 4.0, diluted with water (600 ml.), and stirred for an additional two hours. The insoluble cuprous mercaptide, containing excess cuprous oxide, was centrifuged and washed twice with cold water, after which

it was suspended in water (300 ml.) containing concentrated hydrochloric acid (3.0 ml.) and saturated with hydrogen sulphide. When the decomposition was complete, the copper sulphide was removed by centrifuging, washed several times with water, and the combined filtrate and washings were evaporated to dryness *in vacuo* in an atmosphere of nitrogen. The dry solid consisted of pure β,β-dimethylcysteine hydrochloride. The yield was 3.54 gm., corresponding to 92% of theory. Calc. for $C_5H_{11}O_2NS$. HCl: C, 32.30; H, 6.47; N, 7.55; S, 17.25; Cl, 19.10%. Found: C, 32.69; H, 6.89; N, 7.58; S, 17.32; Cl, 19.45%.

β,β-Dimethylcysteine hydrochloride gave positive ferric chloride, ninhydrin, and nitroprusside tests.

Acknowledgment

The authors are indebted to Mrs. D. Jewitt of our micro-analytical department for the analytical values reported, and to Dr. Paul Trussell and Dr. Olga Volkoff of our micro-biological laboratories for the antibiotic tests. Our thanks are also due to Mr. H. Fleischhacker for technical assistance.

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ON THE SPECIFICITY OF DYE TITRATION FOR ASCORBIC ACID[1]

By Jules Tuba[2], George Hunter[3], and Helen Ruth Steele[4]

Abstract

The 2,6-dichlorophenolindophenol method of assay of vitamin C has been critically examined with respect to a number of fresh, boiled, and otherwise treated plant materials. Comparative values are given for ascorbic acid as determined by the usual method, for non-vitamin C reductants, and for dehydroascorbic acid.

In the fresh uncooked materials examined, non-vitamin C reductants were not found and only in cabbage, peas, and pigweed leaves did dehydroascorbic exceed 10% of the ascorbic acid value.

Boiling produced significant amounts of non-vitamin C reductant in cabbage and peas.

In rose hips the dye titration gives an accurate measure of ascorbic acid. The amounts of dehydroascorbic acid and non-vitamin C reductant, even under storage at room temperature for over a year, are insignificant.

It is concluded that the 2,6-dichlorophenolindophenol method of assay of vitamin C is highly specific but in the case of certain cooked foodstuffs non-vitamin C dye reductants may, without check by other methods, introduce significant errors.

Introduction

Birch, Harris, and Ray (2) in their parallel chemical and biological assays of a wide variety of plant and animal materials dispelled much of the confusion that previously existed on the subject of the specificity for vitamin C of the Tillmann's titration with the redox indicator 2,6-dichlorophenolindophenol. Cysteine was the only substance that might be found in biological material observed by these workers to reduce the dye under the conditions employed. They concluded that the titration method gives accurate results for vitamin C in a wide variety of animal and vegetable materials.

During the last few years the specificity of the dye titration has been called in question from a number of directions. The increase in titration value sometimes found after cooking, the suggested presence of "combined ascorbic acid" (6), the occurrence of dehydroascorbic acid in fresh and stored fruits and vegetables, are all problems that have been exhaustively investigated by Harris and Olliver (3), with the conclusion that the dye titration "can be recommended with confidence for all ordinary routine analyses of plant materials, as giving the total anti-scorbutic activity".

Since the above general conclusion was formulated, observations of non-vitamin C reductants of 2,6-dichlorophenolindophenol compel the attention of those using this dye for the assay of the vitamin.

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Mapson (7) has shown, by use of the formaldehyde method of Lugg (5), that in fresh fruits and vegetables, and in various preserves of these, the dye titration is usually better than 90% due to the ascorbic acid, but in certain dried vegetables, particularly those with a high sugar content and stored at temperatures over 25° C., there may be no ascorbic acid present when the direct dye titration would indicate as much as 93 mgm. per 100 gm. Mapson believes that some of these non-vitamin C reductants are reductones.

The possible occurrence of reductones and related substances has been studied by Wokes and his associates (12), who (13) coined the current misleading term, "apparent vitamin C", to include such substances and others that reduce the dye but possess no antiscorbutic activity.

Various methods, including Lugg's, have been used to differentiate ascorbic acid from such reductants. The method of Levy (4), depending on the fact that ascorbic acid does not reduce the dye in 20% hydrochloric acid solution, while a gluco-reductone solution does, is the only one in addition to Lugg's that we are concerned with here. Levy's method indicates that nearly 20% of the usual dye titration in fresh orange juice is traceable to non-vitamin C reductant(s).

Although there is yet great discrepancy in the observations of different workers on the subject, the trend of the evidence is that non-vitamin C reductants may give rise to considerable error in the usual dye titration in certain foodstuffs that have been dried, baked, or stored for long periods at fairly high temperatures. The work of Wokes and his associates would indicate that there may be appreciable non-vitamin C reductants in certain fresh fruits, particularly green walnuts.

We were led to the present investigation primarily from our interest in rose hips. It seemed that the very high assay given by the dye method in this material should be checked from every possible angle. A biological assay has already been reported (9) showing that the reduction of 2,6-dichlorophenolindophenol by rose hip powder is due wholly to ascorbic acid. In addition, in a paper (10) that accompanies this one, we describe the isolation of ascorbic acid from rose hips as quantitatively as might be expected. We here report our observations on the question of non-vitamin C reductants in relation to the 2,6-dichlorophenolindophenol titration on rose hip preparations and certain other fruits and vegetables. The place of dehydroascorbic acid is also considered.

Experimental

I. *Non-vitamin C Reductants*

The formaldehyde method of differentiating non-vitamin C reductants from vitamin C was first developed by Lugg (5) and later modified by Mapson (7) and by Wokes *et al.* (14). The basis of the use of formaldehyde is, according to Lugg, the fact that at pH 3.5, ascorbic acid will condense readily with formaldehyde, thus becoming non-reducing towards 2,6-dichlorophenolindophenol, while reductones do not combine with formaldehyde at this pH.

Mapson found, however, that there is no pH at which formaldehyde condenses with ascorbic acid without some condensation occurring simultaneously with non-vitamin C reductants. He recommends pH 2, for while the condensation with vitamin C occurs more slowly, the reaction with non-vitamin C reductants proceeds to a lesser degree than at higher pH values such as 3.5. Wokes suggests pH 4.5 for the condensation. While there seems to be general agreement about certain details of the formaldehyde method, such as the optimal concentration of formaldehyde to be used, it was felt necessary to determine which of the various suggested pH values should be chosen for our work.

We followed the rate of condensation of formaldehyde with ascorbic acid alone and, similarly, with "gluco-reductone" solution prepared by the method of Mapson (7). The results in Fig. 1 indicate that 2 is the most desirable pH value to use. In this matter, as in most details of the formaldehyde treatment of material being assayed for non-vitamin C reductants, we have adhered to the method outlined by Mapson, with the titration temperature maintained at 20° C.

Mixtures of ascorbic acid and gluco-reductone were treated with 8% formaldehyde at pH 2.0. Aliquots were titrated, for the first 20 min., at 5 min. intervals and subsequently at 10 min. intervals, up to 120 min. The titration curve is shown in Fig. 2. It will be seen that ascorbic acid is condensed with formaldehyde in 50 to 60 min., and after that the reaction with reductone, which is much slower, gives a linear curve. If the linear part of the curve is then extrapolated to zero time, the value of the intercept on the axis, representing the original amount of the reductone present, should, on subtraction from the total titration at zero time, give the amount of ascorbic acid that was originally present in the mixture of ascorbic acid and reductone, The amount of ascorbic acid calculated from the curve in Fig. 2 agrees to within 1.2% of the amount actually added to the mixture.

Similarly, model experiments were carried out using the differential titration method of Levy (4). To a solution of gluco-reductone, ascorbic acid was added to a concentration of 0.1 mgm. per ml. (*a*) Titration of the mixture gave the dye equivalent of both ascorbic acid and gluco-reductone, since under these conditions both substances reduce the dye. (*b*) To an aliquot of the mixture 36% hydrochloric acid was added in the ratio of 2 parts of solution to 2.5 parts of acid to give a final concentration of 20% hydrochloric acid. Under these conditions, Levy states, ascorbic acid is in its keto form, and will not react with the dye, although the reductone will. Hence titration of this second solution gives the amount of the non-vitamin C reductants. (*c*) An aliquot of (*b*) was diluted with five volumes of water making keto-enol tautomerization again possible. As a result ascorbic acid as well as the gluco-reductone was able to reduce the dye, and a dye equivalent equal to (*a*) was

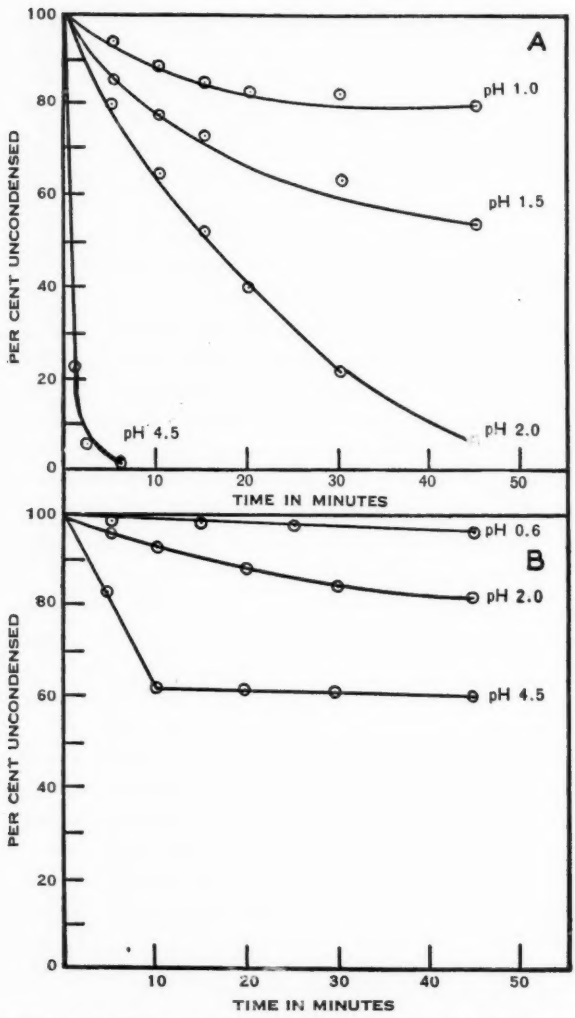

FIG. 1. *The effect of pH on the rate of condensation of formaldehyde with (A) ascorbic acid and (B) gluco-reductone. Temperature, 20° C.*

obtained. The results of the experiment are summarized below and are given in terms of 1 ml. of (*a*), the original solution, containing ascorbic acid = 0.100 mgm. per ml. and gluco-reductone = 0.336 mgm. per ml.

(*a*) Dye equivalent = 0.436 mgm. per ml. reducing substances.
(*b*) Dye equivalent = 0.325 mgm. per ml.
(*c*) Dye equivalent = 0.436 mgm. per ml.

$a - b = 0.111$ mgm. of ascorbic acid, or an error of 11.0%.

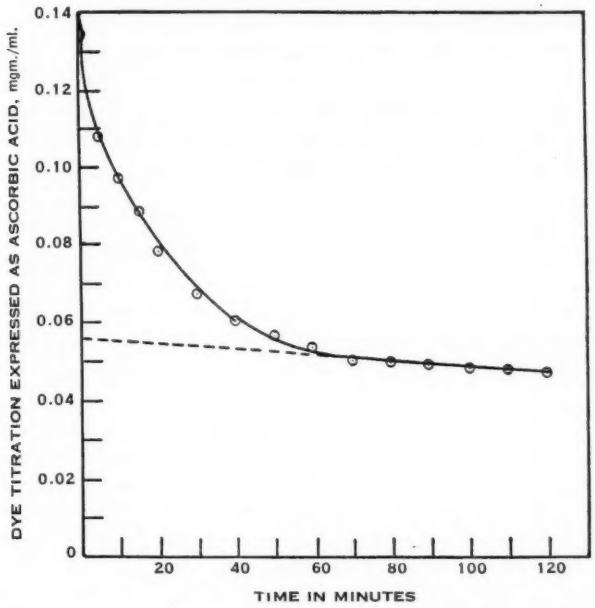

FIG. 2. *Reaction of 8% formaldehyde at 20° C. and pH 2.0 with a solution containing 0.0775 mgm./ml. ascorbic acid and 0.0568 mgm./ml. gluco-reductone. Ascorbic acid found by extrapolation to zero time = 0.1344 − 0.056 = 0.0784 mgm./ml. Error = 1.2%.*

This experiment indicates that it is possible by the method described by Levy to determine with fair accuracy the amounts of ascorbic acid and gluco-reductone in mixtures of the two substances.

ASSAYS OF PLANT MATERIALS

(*a*) *Formaldehyde Method of Mapson* (7)

The assay material is extracted with 5% metaphosphoric acid to give a final concentration of approximately 0.1 mgm. ascorbic acid per ml. or its equivalent in dye reducing substances. The indophenol titration value of the extract is determined and an aliquot is set in the dark for one hour. A decrease of the titration value by the end of that time is due to the presence of dihydroxymaleic acid or certain other compounds found in malt, etc. (7). (This did not occur with any of the materials assayed by us.)

The main part of the extract is brought to pH 2.0 by the addition of sodium citrate and kept at 20° C. for the duration of the determination. An aliquot is titrated, and this value, corrected for the dilution due to formaldehyde, and expressed as ascorbic acid in mgm. per ml. is the initial value at zero time on the graph.

Formaldehyde (36%) is added to the solution to a final concentration of 8%. Aliquots are taken at five minute intervals for 20 min. and titrated

immediately. Thereafter titrations are made every 10 min., generally for another 90 to 100 min. The values of the titrations with 2,6-dichlorophenol-indophenol, expressed in mgm. of ascorbic acid per ml., are plotted against time as the abscissa. The linear part of the curve is extrapolated to the ordinate, and the value thus obtained is that of the non-vitamin C dye reductants in mgm. per ml. (Fig. 3, *A* and *B*).

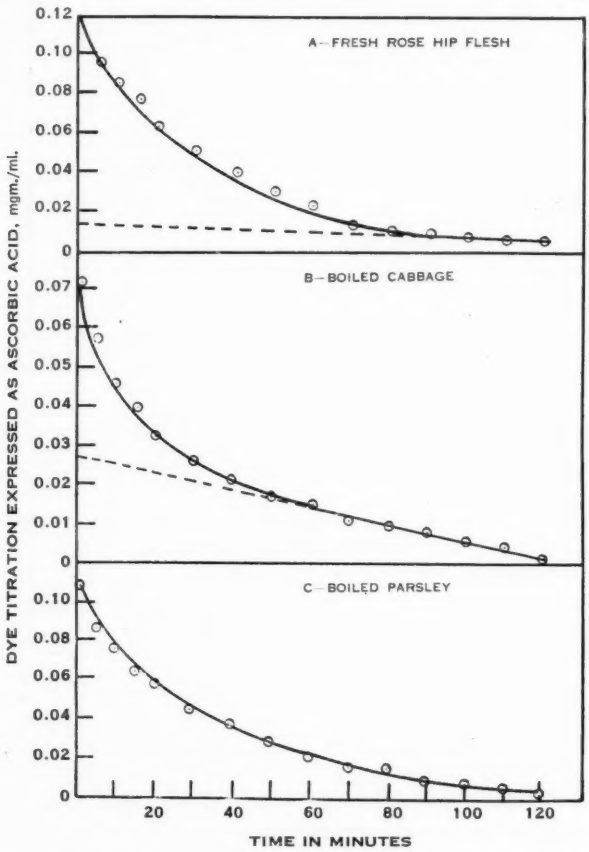

FIG. 3. *Estimation of non-vitamin C reductants by the formaldehyde method in (A) fresh rose hip flesh, (B) boiled cabbage, and (C) boiled parsley.*

One of our chief criticisms of the formaldehyde method, in addition to the time required, is, that while the linear region of the curve is very distinct with model experiments using simple mixtures of ascorbic acid and gluco-reductone (Fig. 2), in many cases it is by no means so definite in assays of plant materials. As a result the slight variations possible in the slope of

this part of the curve (Fig. 3, *C*) will make very appreciable differences in the value of the intercept on the ordinate.

(*b*) *Differential Titration Method of Levy* (4)

The assay material is extracted with 5% metaphosphoric acid in the usual manner. Aliquots are titrated as described to get the combined value of the ascorbic acid and the non-vitamin C reductants. To a portion of the extract 2.5 parts of concentrated hydrochloric acid is added for every 2 parts of the metaphosphoric extract, and the dye equivalent of the solution gives the value of the non-vitamin C reductants. Part of the strong acid solution is diluted with five volumes of water and the reduction of the dye by this solution is due to both vitamin C and non-vitamin C reductants. The first and last titration values should check. Subtraction of the second titration value gives the concentration of ascorbic acid. Results by this method are shown in Table I.

This method has a time saving advantage over the formaldehyde estimation. After the addition of concentrated hydrochloric acid the extract must be titrated at once, otherwise the titration value will alter markedly. In some cases the addition of the concentrated hydrochloric acid turns the extract brown, masking the titration end-point. This occurred in the assay of white currant jelly, where the result by the Levy method differed greatly from the formaldehyde method. Further, the Levy method is not satisfactory with coloured extracts requiring the McHenry and Graham method of titration (6), owing to the rapid fading in the chloroform layer of the pink colour marking the end-point.

II. *Dehydroascorbic Acid*

The amount of the oxidized form of vitamin C in plant material we determined by three different methods. The first of these is that used by Waagen and Pett (11), which consists simply in adding powdered zinc to the acid extract. The difference in titration before and after adding the zinc was considered by Waagen and Pett to be a measure of the amount of dehydroascorbic acid. The second is the commonly used hydrogen sulphide method devised by Bessey (1). Finally, we used the more recent 2,4-dinitrophenylhydrazine method of Roe (8). Apart from being an independent colorimetric method for dehydroascorbic acid, Roe's method has the added advantage that thiourea stabilizes ascorbic acid. It should be noted that Roe considers that the indophenol titration is reliable if dehydroascorbic acid is measured also. He limits the possible interference of reductones to those foods that have been cooked at alkaline pH's because he finds that these interfering substances do not form at acid or neutral reactions.

Discussion

Table I shows comparative values obtained for ascorbic acid by the usual dye titration, non-vitamin C reductants determined by the Mapson and the Levy methods, and dehydroascorbic acid as determined by reduction with

TABLE I

COMPARATIVE ANALYSIS OF PLANT MATERIALS FOR ASCORBIC ACID, NON-VITAMIN C REDUCTANTS, AND DEHYDROASCORBIC ACID. UNLESS OTHERWISE INDICATED ASSAY MATERIAL IS FRESH

Material	Ascorbic acid by dye titration, mgm./ 100 gm.	Non-vitamin C reductants, % of indophenol value		Dehydroascorbic acid, mgm./100 gm.		
		Mapson	Levy	Zinc	Bessey	Roe
Black currants	156.6	0.0	—	25.7	26.8	3.1
Black currant jelly	19.0	6.2	—	—	—	—
Broad beans	10.9	0.0	—	2.7	5.7	1.5
Broad beans, boiled	25.3	0.0	—	1.7	1.2	4.0
Cabbage, sample No. 1	84.9	0.0	—	−30.0	7.5	13.6
Cabbage, boiled, sample No. 1	40.9	20.0	—	−24.3	1.1	3.1
Cabbage, sample No. 2	58.3	0.0	—	−18.4	0.0	9.1
Cabbage, boiled, sample No. 2	38.9	36.0	—	−23.7	2.2	5.7
Cherry plum	0.12	—	—	2.6	2.3	5.0
Cherry plum, boiled	4.4	10.0	—	2.7	5.1	1.9
Guava	87	0.0	0.0	—	—	—
Hazel nut leaves	394.8	2.0	—	13.5	16.2	—
Parsley	315.0	5.0	—	0.0	18.7	16.8
Parsley, boiled	233.3	14.0	—	0.0	6.2	23.5
Parsley, dried	832.0	—	6.0	—	—	—
Peas, sample No. 1	0.4	—	—	22.8	—	20.0
Peas, sample No. 2	0.7	—	—	24.3	—	—
Peas, sample No. 3	2.7	—	—	6.1	—	—
Peas, boiled, sample No. 2	19.8	14.0	—	4.5	3.4	3.1
Peas, boiled, sample No. 3	17.3	15.0	—	1.1	1.5	2.7
Pigweed leaves	275.4	0.0	—	0.0	0.0	—
Pigweed leaves, dried	385.0	0.0	—	27.0	—	53.5
Raspberries	22.4	0.0	—	1.6	1.5	2.5
Raspberry jam	2.7	22.0	—	—	—	—
R. acicularis dried powdered flesh (freshly prepared)	4925	8.0	6.0	0.0	0.0	102.5
R. acicularis dried powdered flesh (stored 16 months)	2902	—	5.0	—	—	—
R. laxa hip flesh	4950	4.0	—	—	—	—
R. woodsii hip flesh	2056	5.0	—	0.0	0.0	26.0
R. woodsii hip flesh	1890	0.0	—	0.0	0.0	18.0
R. woodsii hip flesh	1802	—	6.0	—	—	—
R. woodsii hip flesh	2184	—	2.5	—	—	—
R. woodsii flesh, boiled	2052	6.0	—	0.0	0.0	3.1
Rose hip and crab jelly	245.5	4.0	—	0.0	0.0	0.0
Rose hip and crab jelly	236	12.8	7.5	0.0	0.0	0.0
White currants	64.8	0.0	—	1.3	2.3	5.0
White currant jelly	8.2	—	65.0	—	—	—
White currant jelly	2.3	30.0	—	—	—	—

zinc, with hydrogen sulphide, and by the colorimetric method of Roe. Fresh, cooked, and otherwise processed plant materials were used.

The method of Levy became available only in the later stages of our work so that comparatively few materials were assayed by it. Our few results indicate that the Mapson and Levy methods give values for non-vitamin C reductants of about the same magnitude.

In the materials examined by us, however, it is only in exceptional cases that the non-vitamin C reductant values appear significant. In all fresh

materials tested the non-vitamin C reductant values are zero or negligible. These observations are in accord with those of Harris and his colleagues.

Increase in titration value as a result of boiling is seen especially in the case of broad beans and peas. In the case of beans the increase after boiling is not accompanied by any shift in the values for dehydroascorbic acid nor for non-vitamin C reductant. In the cases of peas the increase is largely accounted for by the appearance of non-vitamin C reductant and an almost corresponding drop in dehydroascorbic acid. Our data on this point, however, are too few to do more than suggest further investigation.

Our results for dehydroascorbic acid by the three methods used are very erratic. With the exception of rose hips there is a measure of concordance between the method of Bessey and that of Roe, but it is not such as to inspire much confidence. On the assumption that the Roe method gives a fair estimate of dehydroascorbic acid, only in the case of fresh cabbage, peas, and pigweed leaves does dehydroascorbic acid exceed 10% of the ascorbic acid. In dried rose hip powder dehydroascorbic acid by one observation amounts to about 2% of the ascorbic acid present. So we may conclude that rose hips contain no significant amounts of either dehydroascorbic acid or of non-vitamin C reductants even after long storage at room temperatures.

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ISOLATION OF ASCORBIC ACID FROM ROSE HIPS[1]

By Jules Tuba[2] and George Hunter[3]

Abstract

A method has been described for the isolation of ascorbic acid from rose hips.

Introduction

Our bio-assay (5) proved that the material in rose hips that reduces 2,6-dichlorophenolindophenol is ascorbic acid. In accordance with this finding is our evidence (7) of the absence of non-vitamin C reductants. Conclusive proof that rose hips are a rich source of vitamin C is now furnished by its isolation.

The high concentration of the vitamin in rose hips might suggest that its isolation should be a simple matter. It has proved otherwise.

The methods successfully used by Szent-Györgyi for its isolation from adrenal tissue, cabbage, and orange (4), and from red pepper (3), are not fitted to separate ascorbic acid from other constituents of rose hips. Likewise the method used by Waugh and King (9) to prepare the vitamin from lemon juice, the method used by Bauman and Metzger (1) for iris leaves and skunk cabbage leaves, and the method of Bukin and Garkina (2) for green walnuts failed to yield any crystalline vitamin when applied to rose hips. We had no difficulty in preparing ascorbic acid from iris leaves by Bauman and Metzger's method.

It required considerable work to overcome the main difficulties in the preparation. We were guided to some extent by our study (6) of the approximate nutrient composition of rose hips. The presence there shown of relatively large amounts of fermentable sugars necessitated either a destruction of these by yeast, or precipitation of the ascorbic acid as a lead salt. In concentrations of the order of 1 mgm. per ml. the lead salt of ascorbic acid begins to precipitate near pH 5.0 and is completely precipitated at pH 7.8. The lead salts of glucose and fructose, even in considerably higher concentration, do not precipitate until the pH greatly exceeds 8.0. By fermenting away the reducing sugars we have succeeded in preparing crystalline ascorbic acid from rose hips without fractionation as lead salt, but it is simpler to dispense with fermentation and use the lead salt in the pH range indicated above.

Separation from the fermentable sugars is, however, a minor difficulty in the preparation from rose hips. If rose hips are treated with aqueous barium acetate as described by Szent-Györgyi for red peppers, and lead acetate added

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Contribution from the Department of Biochemistry, University of Alberta, Edmonton, Alta.
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[3] *Professor of Biochemistry.*

to the filtrate, an enormous lead precipitate is obtained in the usual pH range. When the lead is removed from this material, subsequent fractionation by means of solvents is futile.

Aqueous extracts from hips contain large amounts of a pectin-like material that precipitates as a lead salt in the ascorbic acid pH range. Much of this material can be removed by preliminary treatment with methanol with negligible loss of ascorbic acid. In the subsequent lead salt, however, there still remains considerable non-ascorbic acid material but this can be fairly readily removed by solvent fractionation.

Bauman and Metzger add lead acetate to a final concentration of 25 to 30%. This is quite unnecessary and, indeed, in the case of rose hip material detracts from the value of the lead fractionation. By using the minimum necessary amount of lead acetate the specificity of the lead fractionation is enhanced.

The necessary amount of lead acetate for a particular batch is quite easily determined in the following way. An estimate of the ascorbic acid in solution is made by titration of a small aliquot portion. A solution of lead acetate is added to give a weight of lead acetate trihydrate about six times that of the ascorbic acid present in the solution. This suffices for a simple aqueous solution of ascorbic acid, as arbitrarily tested by adding an excess of ammonia, centrifuging, and proving the absence of ascorbic acid by dye titration on the acidified supernatant fluid. With rose hip extracts some three to five times this amount of lead acetate may be necessary, but the precise amount can be determined with a few trials on small aliquot portions as above indicated.

The preparation may now be described.

Preparation

Material

Ripe rose hips were just covered with water and boiled till soft. They were stirred and crushed to a mixture of pulp and seeds. The seeds were separated by passing through a sieve and the resulting "puree" was transferred to beer bottles, sterilized, and capped. This material, resembling thin tomato catsup, was stored for over a year without loss of vitamin. It usually contained 3 to 4 mgm. of ascorbic acid per ml. and served as our starting material for the preparation.*

Five hundred millilitres of puree (1400 mgm. of ascorbic acid) was reduced *in vacuo* to a paste (without crusting on the sides of the flask). Absolute methanol was added with shaking until no more appreciable precipitation took place (four to five volumes). The material was filtered through a Büchner. The filtrate was reduced *in vacuo* to a small volume, while the residue on the Büchner was resuspended in methanol and again filtered. The washing was repeated and the two last filtrates added to the thin syrup

** Although our work was done chiefly with such purees, we were able to isolate crystalline ascorbic acid from dried, powdered rose hip flesh, containing 3.5 to 5.0% of ascorbic acid. Such material was extracted and washed twice with methanol, and the extract and washings were treated as in the case of the puree.*

from the first filtrate. A further precipitate appears here and is removed. The filtrate is reduced *in vacuo*, the residue dissolved in water, and made to one litre volume (1200 mgm. of ascorbic acid).

Glacial acetic acid is added to the solution to pH about 3.5. The predetermined amount of 40% lead acetate* (60 ml.) is added and the solution mixed. Its pH will now be near 4.5. The precipitate is removed, washed, and discarded (with loss of about 50 mgm. of ascorbic acid). To the filtrate strong ammonia is added to pH 7.8, the precipitate collected in centrifuge bottles, and the supernatant fluid and washings are discarded (with loss of about 50 mgm. of ascorbic acid).

Crushed ice is added to the precipitate in a 250 ml. centrifuge bottle and the precipitate stirred to a smooth thick paste, in presence of ice.

Ten per cent sulphuric acid is added until the solution gives a clear blue test with Congo red paper; the end-point is checked with barium chloride.

The lead sulphate precipitate is removed, the filtrate collected, and an aliquot titrated (905 mgm. of ascorbic acid).

The solution is reduced *in vacuo* to a thick syrup. It is dissolved in a minimum amount of anhydrous methanol with warming. Then five volumes of anhydrous acetone is added gradually with shaking and cooling. Much of the gummy precipitate adheres to the flask and the rest is centrifuged. The precipitates are dissolved in a minimum amount of methanol in the centrifuge bottles. This is transferred to dissolve the main bulk in the flask. Five volumes of acetone is added. The resulting gum may be treated again with methanol and acetone: usually two such washings are sufficient to remove almost all of the vitamin.

The methanol–acetone extract and washings are combined and reduced to a small volume *in vacuo*. The syrup thus obtained is dissolved in a minimal amount of methanol and 10 volumes of acetone is added. The precipitate is again washed twice with methanol and acetone. The combined methanol–acetone extract and washings are again reduced *in vacuo* to a syrup.

The syrup obtained after the above fractionation with methanol and acetone is dissolved in the least amount of anhydrous methanol. Then 10 volumes of anhydrous acetone is added and finally 10 volumes of anhydrous petroleum ether. There is formed a large amount of oily precipitate, some of which remains suspended in the supernatant liquid, and is separated by centrifugation. The oily precipitate formed at this step tenaciously holds the vitamin, and the procedure has to be repeated six to eight times with methanol, acetone, and petroleum ether in the above ratio, to get nearly quantitative recovery (at this stage 850 mgm. of ascorbic acid).

** A 5 ml. aliquot is removed, 0.1 ml. of 40% lead acetate added and, after mixing, ammonia is added until the solution is alkaline to phenolphthalein. The mixture is centrifuged and 1 ml. of the acidified supernatant fluid is titrated with standard dye. If there is an appreciable titration another 5 ml. aliquot is tested with 0.2 ml. of 40% lead acetate and the procedure repeated until there is a negligible titration.*

The syrup remaining after the fractionation with methyl alcohol, acetone, and petroleum ether is dissolved in a small amount of acetone and transferred to a boiling-tube. The tube is then evacuated until much of the acetone is removed and the syrup is moderately thick. The evacuated tube is placed in an ice–salt mixture. Crystallization may start at once, and is allowed to continue for one to two days. The crystals are filtered off and washed in cold anhydrous acetone. Yield; 560 mgm. acetone derivative or 456 mgm. ascorbic acid or 33% of that originally present. Crude product: melting point, 193° to 195° C. $[\alpha]_D^{20°}$, in absolute ethanol = + 13. The corresponding values recorded by Vargha (8) for the monoacetone derivative of ascorbic acid are 220° to 222° C. and + 15.

The dye titration yielded values corresponding to the composition of the monoacetone derivative.

The crude and slightly brown monoacetone derivative was dissolved in water and boiled with a little charcoal. The filtrate was dried in a vacuum desiccator and the residue recrystallized from ethanol.

The crystals are proved to be ascorbic acid by the following properties.

Melting point, 182° to 186° C. (Authentic, 184° to 186° C.)

$[\alpha]_D^{20°}$, in water, + 21 (Authentic, + 22.5)

$[\alpha]_D^{20°}$, in absolute ethanol, + 57 (Authentic, + 58.7)

Remarks

From our experience in various preparations we believe that, for all practical purposes, all the dye reducing power of rose hips is attributable to ascorbic acid.

From the boiled pulp the reducing material is quantitatively extractable by water and methyl alcohol so that we can conclude the vitamin is "free" in the tissue. At no stage in the preparation was there any ground for supposing the presence of any non-vitamin C reductant.

Appreciable loss in the course of any preparation of vitamin C without recourse to maintaining anaerobic conditions is to be expected. At certain stages in the method described above there can be great losses if adequate precautions are not taken. In the initial stage, there is fairly rapid destruction of the vitamin in aqueous methyl alcohol solution and the "puree" extract should be reduced to the gum stage as quickly as possible. The syrups or gums can be kept almost indefinitely without loss of vitamin.

The other stage at which there can be great loss of the vitamin is in freeing it from its lead salt. The sticky lead precipitate is hard to disperse in cold water and recourse to violent shaking, after the addition of some sulphuric acid, incorporates oxygen with resultant destruction of the vitamin, especially perhaps in presence of traces of catalyst, like copper.

In the subsequent solvent fractionation there is little destruction of the vitamin. Losses that do occur are traceable to incomplete washing of residues.

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